Merkel cell polyomavirus-specific CD8 T cells in Merkel cell carcinoma: T cell receptor diversity & novel immune therapies

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Abstract

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Merkel cell carcinoma (MCC) is an aggressive skin cancer with a disease-specific mortality of 46%. Unfortunately, no FDA-approved treatments for advanced disease exist. Over 80% of MCCs are caused by persistent expression of T-antigen oncoproteins from the Merkel cell polyomavirus (MCPyV). Systemic immunity is strongly linked to both MCC incidence and disease-specific survival, with an especially important role played by the adaptive immune response. Additional background about the biology and immune response to MCC is provided in the Introductory **Chapter 1.** The current standard of care for advanced MCC, cytotoxic chemotherapy, elicits tumor responses in approximately half of patients, but responses are not durable with a median progression-free survival among responders of only 3 months. In **Chapter 2**, we review the literature surrounding such conventional therapeutics and outline the scope of emerging immune-based therapies.

Immune-stimulating agents are thought to hold great therapeutic promise for MCC. Over half of patients with MCCs caused by MCPyV ('virus-positive MCC') develop robust oncoprotein-specific antibodies and/or T cell responses to persistently expressed T antigen (T-Ag) of MCPyV. However, though many patients have cytotoxic CD8+ T cells specific for these oncoproteins, MCCs still develop and persist. Previous studies found that robust infiltration of

CD8+ lymphocytes into the tumor is associated with 100% MCC-specific survival, yet unfortunately infiltration is observed in only 4-18% of MCCs. We explored two distinct mechanisms that we hypothesized may contribute this observed immune dysfunction. Importantly, we hoped to uncover ways in which this immune evasion may be therapeutically targeted.

First, we explored the hypothesis that there is significant genetic and functional diversity among T cell receptors (TCRs) recognizing one common HLA type/MCPyV epitope, and that TCR repertoire differences may be linked to patient outcomes. Indeed, detailed in **Chapter 3,** we found almost 400 different TCRβ clonotypes among epitope-specific T cells from 12 patients. Only *one* of these unique TCRβ clonotypes was detected in more than one patient, highlighting the diversity of the T cell response to this epitope. Additionally, there is significantly improved MCC-specific survival among patients with increased infiltration of epitope-specific T cells within their primary tumors. By studying the functional properties associated with individual TCRs among select patients, we found that patients with improved MCC-specific survival have more functionally avid TCRs. This work has identified avid TCRs with potential for use in transgenic T-cell therapy for MCC.

Secondly, we hypothesized that MCPyV-specific T cells are exhausted. In **Chapter 4** we found that MCPyV-specific peripheral blood mononuclear cells (PBMC) and tumor infiltrating lymphocytes (TIL) express high levels of inhibitory receptors that inhibit TCR signaling (PD-1 and Tim-3) directly *ex vivo*. In addition, we show that while PBMC do not produce the effector cytokine IFN-γ in response to viral peptide stimulation directly *ex vivo*, they are able to regain effector function after culture, which is enhanced by the addition of antibodies that block these inhibitory receptors such as anti-PD-1.

Remarkably, since the review of emerging immune-stimulating agents (**Chapter 2**) was published in Spring of 2013, many of these novel immune-stimulating agents have now been

evaluated in formal studies and have showed impressive efficacy. A retrospective analysis of the use of single-fraction radiation therapy in metastatic MCC patients is presented in **Chapter 5**, demonstrating the 94% efficacy at palliating target tumor lesions with few side effects and impressive durability, especially among patients with intact systemic immunity.

In addition, PD-1 blocking antibodies are now being utilized therapeutically, and in **Chapter 6** we present the results of the first clinical trial of a PD-1 agent, pembrolizumab, in MCC. Excitingly, 54% of MCC patients in the trial responded to this drug, and 67% of responding patients remained progression-free at 6 months. In this chapter we also detail our finding that both virus-positive and virus-negative MCC respond to pembrolizumab, and that many of the biomarkers used for other cancers to predict response to PD-1 agents, such as tumoral PD-L1 expression or CD8 infiltration, may not be relevant in MCC. We further perform correlative studies on patient tumor and PBMC samples from these clinical trial patients throughout their therapeutic course, and our preliminary findings are presented in **Chapter 7.**

In summary, we have used our extensive and unique repository of clinically annotated MCC blood and tumor specimens to further elucidate aspects of the CD8+ T cell response to MCC such as TCR diversity, inhibitory mechanisms, and augmentation with immunotherapy. These studies have helped us define the mechanisms by which some patients mediate superior disease outcomes, and have contributed to our goal of improving therapeutic options for patients with advanced MCC.

Dedication:

To my family and fellow ladies of science, with love.

Acknowledgments:

First, I would like to thank Dr. Paul Nghiem, without whom this work would not have been possible. I embarked upon graduate school with the expectation of gaining scientific skills, but have been pleasantly surprised that the majority of what I've learned from Paul has been so broadly applicable and personally transformative. These skills- the ability to communicate effectively, the benefits of collaboration, and the power of being relentless- will serve me well in my career as a physician scientist. I hope my path in his footsteps will accomplish a fraction of what he has already done for patients with MCC.

My 'second PI,' Dr. David Koelle, has provided me with extremely detailed, timely, and brilliant feedback on the nitty-gritty of T cell biology. His help and the skills I gained by working in his lab were essential for the success of my thesis projects.

I also could not have accomplished this work without the help of my colleagues in the laboratories of Drs. Nghiem and Koelle, who provided scientific and moral support for the past four years. I will treasure memories of each and every pastry break. I specifically thank Dr. Candice Church for backing me up scientifically at every step of the way.

Lastly, I wish to express my deepest appreciation for the patients with MCC who have donated the blood and tumor tissue that was invaluable for these studies.

Table of Contents

1. Introduction to Merkel cell carcinoma (MCC), the Merkel cell polyomavirus (MCPyV), and the immune response to MCPyV

Merkel cell carcinoma is an often-lethal skin cancer without durable treatments for advanced disease. Merkel cell carcinoma (MCC) is a highly aggressive skin cancer associated with UV exposure, advanced age, immune suppression, and the Merkel Cell polyomavirus (MCPyV) in at least 80% of cases[1-3]. MCC incidence in the US is 2000 cases each year, and the disease-specific mortality is three times that of malignant melanoma (46% vs 15%)[4,5]. There are no FDA-approved agents to treat this cancer, and there is a paucity of effective treatments for advanced disease. Patients with distant metastatic disease have a 5-year survival rate of 0-18% with a median of only 9.6 months from diagnosis of initial metastasis to death[6,7] (**Figure 1**). Chemotherapy is the current standard of care for patients with advanced disease, and while approximately half of patients initially respond to this treatment, responses are not durable with a median time to progression of only 94 days[8], highlighting the need for improved therapies to treat this cancer. In **Chapter 2** of this dissertation, we review conventional therapeutics for advanced MCC and outline the scope of immune-based therapies that were newly emerging as of Spring 2013. Excitingly, many of these novel agents have now been evaluated in formal studies, some of which are included in this thesis. A retrospective analysis of single-fraction radiation therapy in metastatic MCC patients is presented in **Chapter 5.** In addition we present results of clinical efficacy for the first clinical trial of an immune checkpoint inhibitor, pembrolizumab, in MCC, as well as correlative studies into the mechanism of action and/or predictors of response for this drug (**Chapters 6 & 7**).

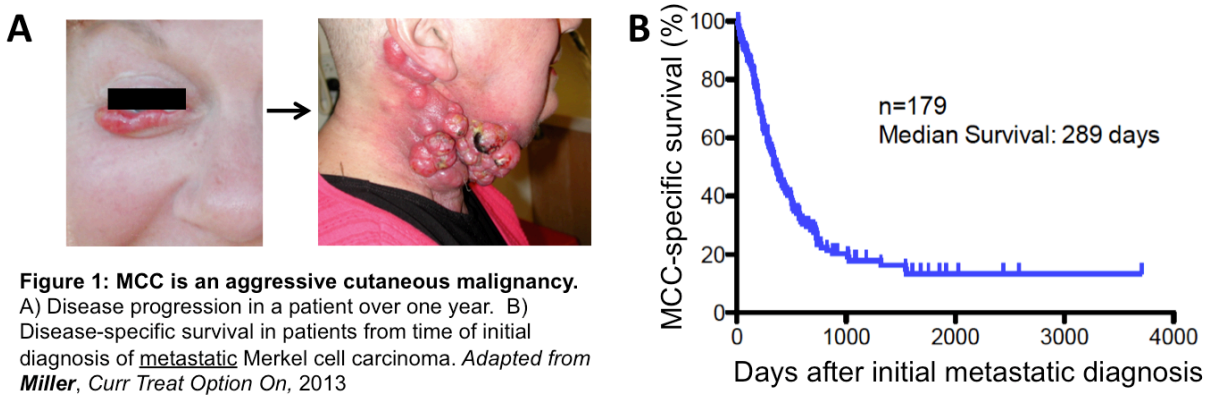

Figure 1: MCC is an aggressive cutaneous malignancy. A) Disease progression in a patient over one year. B) Disease-specific survival in patients from time of initial diagnosis of metastatic Merkel cell carcinoma. *Adapted from* ***Miller***, *Curr Treat Option On,* 2013

MCPyV is a common virus that can, in rare circumstances, cause MCC. MCPyV is a double-stranded non-enveloped DNA virus with a small genome encoding capsid Viral Proteins 1 & 2 (VP1 and VP2) and small and large T-antigens (T-Ag). Infection with MCPyV is common; MCPyV can be detected in skin swabs on 60-80% of healthy volunteers[9] and 60% of the general population is seropositive for antibodies against VP1 & 2 by age 20[10]. However, infection is asymptomatic in most cases. MCC develops only in the confluence of several rare events, including local or systemic immune suppression in combination with two mutations in MCPyV that 1) allow integration of the virus into the host genome and 2) truncate the oncogenic Large T-Ag[11] **(Figure 2**). This truncation mutation eliminates expression of the T-Ag viral helicase domain that would otherwise enable viral replication, lysing host cells.

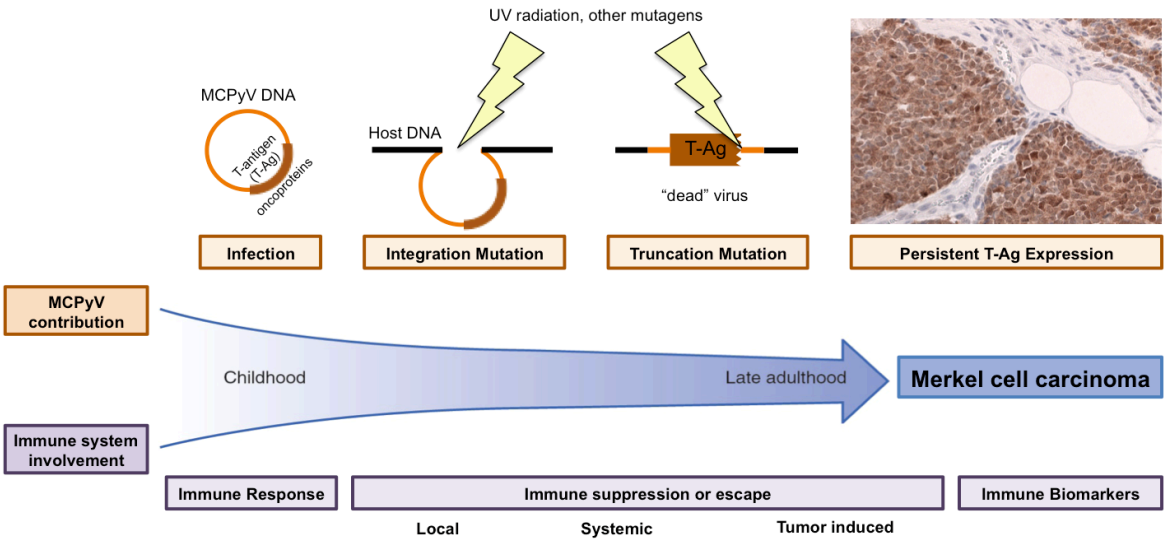

Figure 2. Although infection with MCPyV is common, a progression of several rare mutagenic events and escape from immune surveillance likely precede the development of Merkel cell carcinoma. Ultraviolet (UV) radiation or other environmental mutagens may mediate virus integration into the host genome and Large T (LT)-antigen truncation mutations. These sequential mutational events result in persistent T-Ag expression (brown stain with IHC anti-LT antibody, CM2B4) that plays a key role in MCC pathogenesis {refs}. *Adapted from Bhatia et al, Current Onc Reports, 2011.*

Many lines of evidence show that MCPyV is causal and not mearly a bystander in the tumorigenesis of virus-positive MCC. The integration site of MCPyV, while variable between patients, is clonal in virus-positive MCCs[1], demonstrating that integration is an early event in MCC pathogenesis. In addition the MCPyV small and large T-Ags, similar to transforming T-Ag in other polyomaviruses such as SV40 T-Ag, contain many domains that may contribute to oncogenesis[12-15]. One mechanism that can contribute to transformation is binding of host tumor suppressor retinoblastoma by the LXCXE domain of Large T-Ag, which results in E2F induction and cell cycle progression[16]. More recently, it has been discovered that the small T-Ag can transform cells *in vivo,* in a process dependent on the LT-Antigen stabilization domain of small T-Ag[17]. Lastly, T-Ag expression is persistently required for maintenance of MCPyV+ MCC cell lines[18].

The immune response plays a critical role in fighting MCC. Multiple studies have linked the immune system with the incidence and prognosis of MCC. The increased incidence of MCC among patients with immunosuppression[3] led to the search for and discovery of MCPyV. While

only 10% of MCC patients have systemic immune suppression, these patients have poorer MCC-specific survival[19,20]. The adaptive immune system can recognize and mount protective responses to MCPyV. Over 40% of MCC patients (compared to only <1% of controls) make antibodies to T-Ag that rise and fall with disease burden[19] and these patients have improved recurrence-free survival[21]. Cellular immune responses are also associated with improved prognosis. Specifically, increased intratumoral CD3+ cell count is an independent prognostic factor of increased survival[22] and intratumoral CD8+ lymphocyte infiltration is associated with 100% survival in MCC, independent of tumor stage at diagnosis[23] (**Figure 3**).

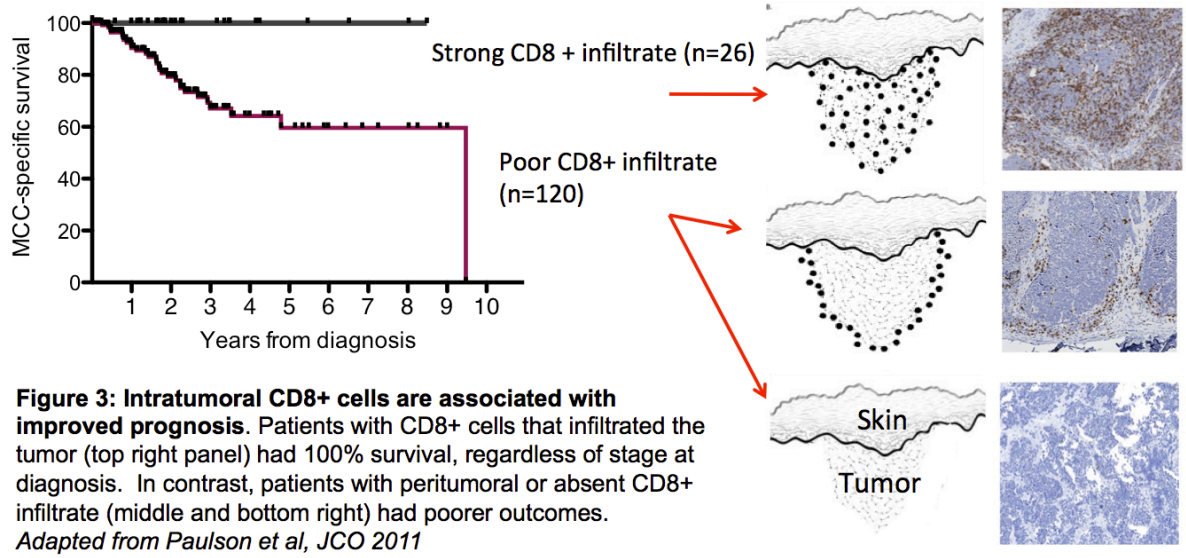

Figure 3: Intratumoral CD8+ cells are associated with improved prognosis. Patients with CD8+ cells that infiltrated the tumor (top right panel) had 100% survival, regardless of stage at diagnosis. In contrast, patients with peritumoral or absent CD8+ infiltrate (middle and bottom right) had poorer outcomes. *Adapted from Paulson et al, JCO 2011*

MCPyV-specific CD4 and CD8 T cells can be identified in blood and tumors from MCC patients. We hypothesized that lymphocytes could recognize and mount an effector response specific to the persistently expressed viral T-Ags. We created a library of overlapping 13 aa peptides using the sequence of the persistently expressed regions of the T-Ags. We combined lymphocytes isolated from cultured tumor infiltrating lymphocytes (TIL) or peripheral blood mononuclear cells (PBMC) with pools of these T-Ag peptides plus autologous antigen presenting cells (APCs) to present peptides to the lymphocytes and measured reactivity via production of interferon-γ (IFNγ). TIL was screened for reactivity using an overnight intracellular

cytokine staining assay and flow cytometry. Production of IFNγ from PBMC directly *ex vivo* was infrequently observed. Therefore, PBMC was instead cultured for 9 days in the presence of peptides before measuring IFNγ secretion via the sensitive ELISPOT assay. This allowed us to detect at least 2 CD4 and 5 CD8 T cell responses specific for MCPyV [24](unpublished results). The assay was repeated to determine the minimal epitope required for T cell stimulation, and HLA-restriction of the epitope was determined using partially HLA-matched APCs.

After determining the minimal epitope and HLA-restriction for each CD8 T cell response, HLA-I tetramers were synthesized. Tetramers consist of four HLA molecules bound to their cognate MCPyV epitope and coupled to a fluorophore to allow sensitive and specific detection of MCPyV-specific T cells via flow cytometry. As detailed in **Chapter 4**, the tetramer composed of HLA-A24 and MCPyV peptide LT 92-101 'EWWRSGGFSF' was used to detect T cells of this specificity in PBMC of 64% of HLA-A24 MCPyV+ MCC patients, but 0% of HLA-matched MCPyV negative patients or control subjects[25] (**Figure 4**). Using tetramers, we are able to track MCPyV-specific T cells and have found that they fluctuate in prevalence in parallel with tumor burden[25].

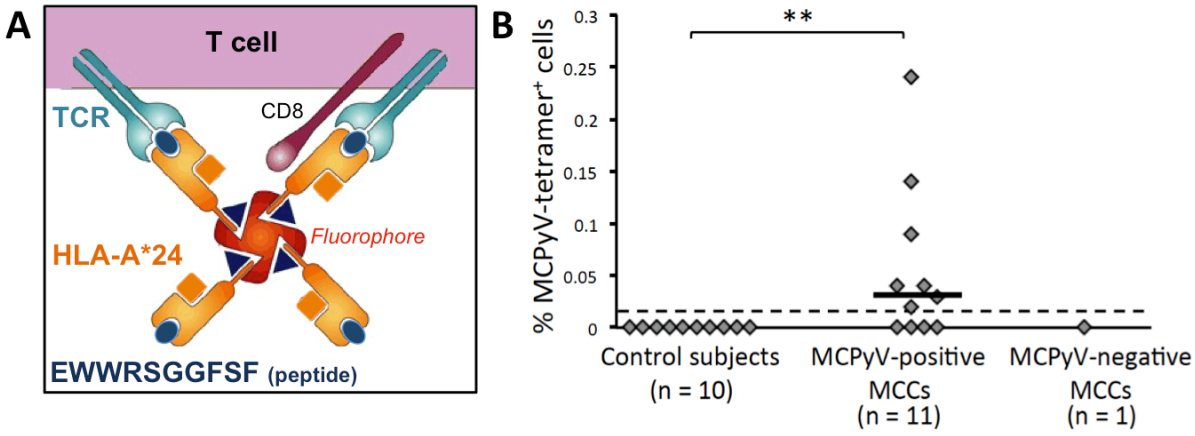

Figure 4: Tetramers label MCPyV-specific CD8 T cells in MCC patients. A) HLA/peptide monomers are conjugated to a fluorophore which allows sorting or analysis of T cells of that specificity. B) MCPyV-specific T-cell frequencies among patients or control subjects. Dashed line indicates the threshold of tetramer detection. Solid line indicates the median among patients with detectable MCPyV-specific T cells. All analyses were from the first available blood draw of subjects who were HLA- A*24 or HLA-A*23 positive. *Adapted from Afanasiev, Yelistratova, **Miller** et al., Clin Cancer Res 2013.*

MCCs employ many mechanisms to avoid destruction by the immune system. Patients develop MCC despite the presence of MCPyV-specific CD8+ T cells, leading us to hypothesize that tumors are able to evade killing by cytotoxic T cells. To date, three main mechanisms of T cell immune evasion have been elucidated in our laboratory: 1) HLA-I is downregulated in the majority (84%) of MCCs (n=114). This common mechanism of evading T cell responses is observed in many cancers. Downregulation can be reversed using agents in clinical use, such as injection of IFN-beta into MCC tumors or a single low dose of radiation[26]. 2) T cells are inhibited from entering the tumor. Endothelial (E)-selectin, a cell adhesion molecule that binds cutaneous lymphocyte antigen on effector T cells and allows their extravasation from blood, is downregulated in 52% of MCCs. Poor E-selectin expression on intratumoral blood vessels is correlated with reduced CD8+ cell infiltration as well as with poorer MCC-specific survival[27]. 3) **MCPyV-specific T cells are exhausted.** T cell exhaustion can occur after chronic unresolved exposure to Ag. This phenomenon was first discovered in the context of chronic viral infections[28-32] and is also noted in non-viral cancers such as melanoma[33]. Exhaustion is associated with increased surface expression of inhibitory receptors that block T cell receptor signaling (including programmed cell death protein 1 [PD-1]), reduced secretion of effector molecules, decreased proliferative capacity, and eventual deletion of Ag-specific T cells (**Figure 5**).

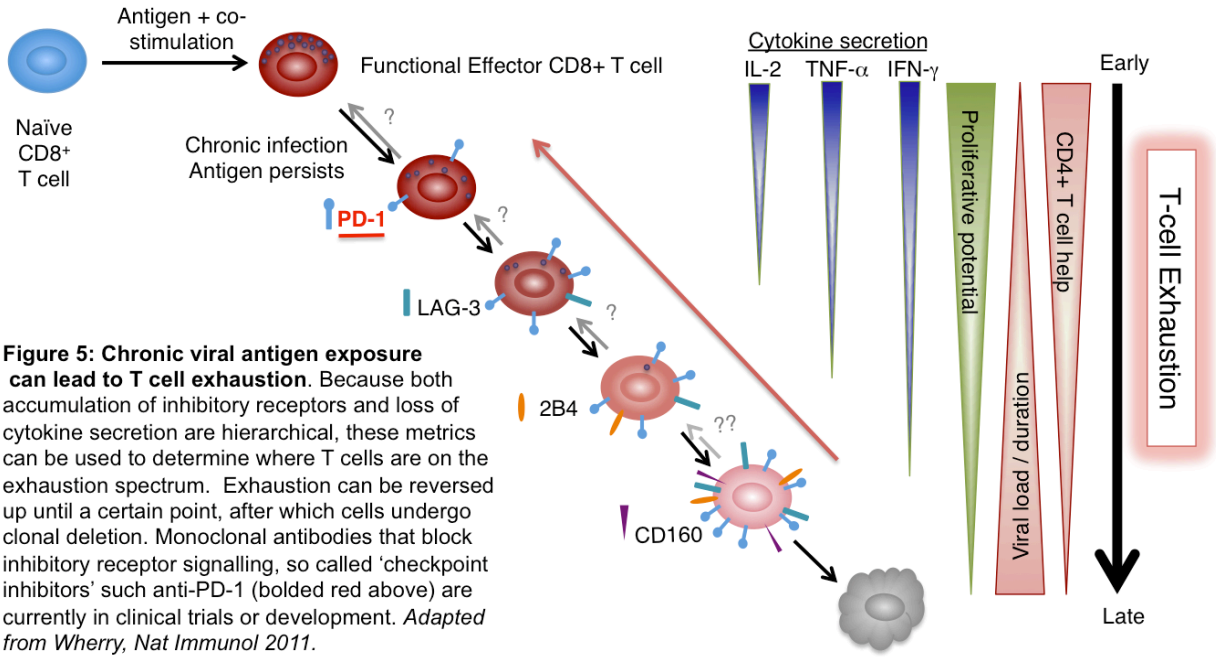

Figure 5: Chronic viral antigen exposure can lead to T cell exhaustion. Because both accumulation of inhibitory receptors and loss of cytokine secretion are hierarchical, these metrics can be used to determine where T cells are on the exhaustion spectrum. Exhaustion can be reversed up until a certain point, after which cells undergo clonal deletion. Monoclonal antibodies that block inhibitory receptor signalling, so called 'checkpoint inhibitors' such anti-PD-1 (bolded red above) are currently in clinical trials or development. *Adapted from Wherry, Nat Immunol 2011.*

We hypothesized that chronic T-Ag exposure exhausts MCPyV-specific CD8+ T cells and reduces their capability to mount a cytotoxic response. In **Chapter 4**, our studies detailed on CD8+ T cells recognizing a single MCPyV epitope provide evidence of T cell exhaustion, as these MCPyV-specific cells have significantly higher expression of two inhibitory receptors compared to T cells in MCC patients specific for other common viruses [25] and have impaired secretion of effector cytokines upon stimulation with MCPyV peptides *ex vivo* [24] (**Figure 6**).

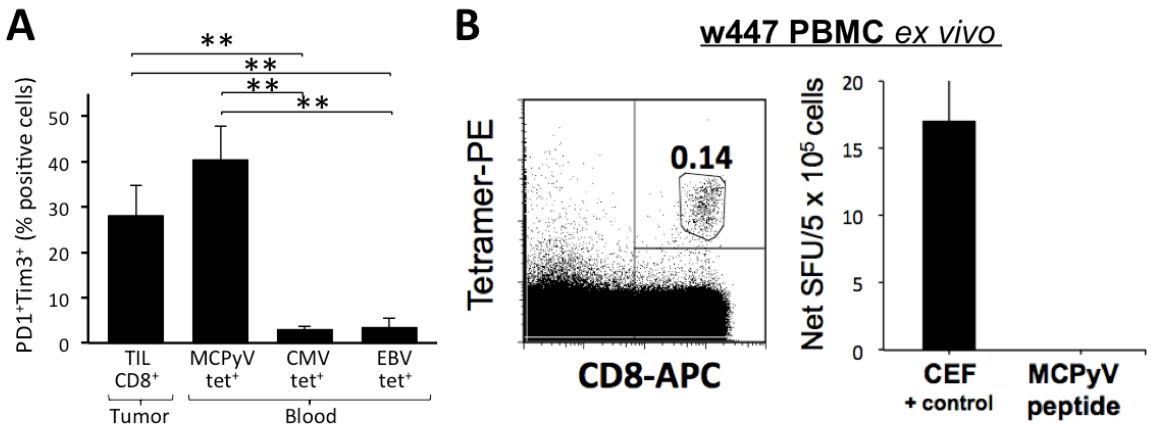

Figure 6: MCPyV-specific T cells are exhausted *ex vivo*. A) MCPyV-specific cells from blood and TIL have increased co-expression of PD-1 and Tim-3, indicative of T cell exhaustion, compared to control virus-specific cells from MCC patients, n= 5-7. **p<0.01. B) Despite the presence of MCPyV-specific T cells, PBMC responds only to positive control peptides (CEF; pool of 32 immunogenic viral peptides) but not to a peptide from MCPyV in an *ex vivo* IFN-γ EliSPOT. In contrast, cultured TIL responds to both control and MCPyV peptides (data not shown). *Adapted from Afanasiev, Yelistratova, **Miller** et al., CCR 2013* (A) and *Iyer/Afanasiev, CCR, 2011* (B).

The immune system can be harnessed for therapeutic use against MCC. Adoptive T cell therapy (ACT) with cancer-Ag-specific CD8+ T cells has led to durable cancer regressions in patients[34-38]. We are executing a Phase I/II Clinical Trial (NCI R0ICA176841, P. Nghiem, PI) to treat MCC patients with autologous MCPyV-specific T cells. PBMC are cultured *ex vivo* for 6 weeks with peptide-pulsed autologous dendritic cells plus cytokines to expand the MCPyV-specific T cells, and to reverse the exhausted phenotype of the T cells before tetramer-sorted T cells are infused. Patients are treated with radiation or IFN-beta to upregulate HLA-I expression on the tumor prior to T cell infusion. Despite high *in vitro* cytotoxicity of infusion product toward target cells and persistence of infused tetramer + T cells in all but one patients' PBMC for several months after infusion, three of the four patients developed progressive disease. To investigate how we might improve efficacy of ACT, we began an in-depth study of the T cell receptors that mediate the interaction between the T cell and tumor.

T cell receptors (TCRs) are remarkably diverse in sequence and binding strength- even when specific for a single antigen. Cytotoxic T cells recognize and bind tumor cells through their MCPyV-specific T cell receptors (TCRs). Most TCRs are heterodimers of one α and one β polypeptide whose genes are formed by somatic recombination of V-J (α) or V-D-J (β) segments that become joined to a constant region. There are numerous isoforms of each V, D and J segment leading to an estimated combinatorial diversity of 10^{18} receptors before thymic selection. The complementarity determining region 3 (CDR3) formed by the V(D)J junction contacts the peptide-HLA antigenic complex and significantly contributes to the specificity and affinity of the TCR for cognate peptide-MHC (pMHC)[39]. Overall strength of multiple TCR interactions with cognate pMHC is classified as avidity.

CD8+ T cells with more avid MCPyV-specific TCRs may be more effective at fighting MCC. Adoptive transfer of CD8+ T cells with high vs. low avidity TCRs for the same pMHC has been used in mice to demonstrate the superiority of more avid TCRs at controlling both HIV viral load[40] and eradicating established melanoma metastases[41]. T cells with avid TCRs are more sensitive and thus recognize targets with low antigen density, initiating target lysis faster[40]. Importantly, the functional advantage conferred by an avid TCR is retained when the TCR is transferred into other cell lines or primary CD8+ PBMC[42]. In human clinical trials of metastatic melanoma with transgenic T cells expressing a high or low avidity TCR to the same pMHC, patients experienced a greater response to therapy with the high avidity TCR (30% vs 13% partial response)[43].

Based on this and other literature, we hypothesized that patients who have MCPyV-specific CD8+ T cells with more avid TCRs will have improved MCC-specific outcomes compared to patients with low avidity T cells. Preliminary work supporting this conclusion is presented in **Chapter 3,** where we generated MCPyV-specific CD8+ T cell clones from blood

and tumor of several MCC patients and characterized the functional avidity of T cell clones with unique TCRs.

In addition, we theorized that highly avid MCPyV-specific TCRs may not only be advantageous in the context of an endogenous immune response to MCC, but could be harnessed for effective T cell therapy using transgenic TCR T cells. The above studies have generated several avid TCRs that will be tested as candidates for creation of such therapeutic T cells.

Therapies that enhance the existing immune response are particularly efficacious in MCC. The importance of the immune system in MCC as well as evidence that MCPyV-specific T cells are exhausted (**Chapter 4** and **Figure 6**) has led to trials of many immune stimulating agents in advanced stage MCC patients. In addition to the adoptive T cell trial discussed above, therapies include 'checkpoint inhibitors,' intratumoral injection of agents that skew the immune system toward a Th1-type response, and single fraction radiation (**Table 1** and reviewed in **Chapter 2**)[44-50].

Table 1: Immunotherapies being used to treat MCC Patients in our cohort

Agent	Target Cell	Mechanism of Action
PD-1	CD8+ T cell	Blocks inhibitory/exhaustion signaling to CD8+ T cells
PD-L1	Tumor, APC	Blocks inhibitory/exhaustion signaling to CD8+ T cells
Adoptive T cell Therapy (± PD-L1)	Tumor	Expansion of and restoration of cytotoxic capability to tumor-targeted lymphocytes
4-1BB	CD8+ T cell	Costimulatory signal for immune activation
IL-12 DNA	Lymphocytes & NK cells	Promotes Th1 response; increases IFN-γ and cytolytic activity
GLA	APC	Toll-like receptor (TLR) 4 agonist
8 Gy SFRT	Tumor	DNA damage, immune stimulation

Adapted from ***Miller*** *et al., 2013* (**Chapter 2**). APC= Antigen presenting cell, SFRT= Single Fraction Radiotherapy.

As mentioned above, chronic viral Ag exposure can lead to T cell exhaustion in which T cells progressively lose effector function and increase expression of surface receptors that inhibit TCR signaling, such as PD-1 (**Figure 4**). Checkpoint inhibitors are monoclonal antibodies that block inhibitory receptor signaling, either by binding to the receptor or to a ligand such as

PD-L1, to restore immune function. This class of drugs has been shown to mediate significant, durable tumor regressions in many human cancers, with fewer side effects than chemotherapy. Agents that block the PD-1/PD-L1 signaling pathway have shown efficacy in a large percentage of patients treated in recent clinical trials, including up to 38% of metastatic melanoma patients[51], 27% of renal and 18% of non-small cell lung cancer patients[51]. Based on the frequency at which MCC patients generate tumor-reactive TIL and the observation that MCPyV-specific T cells express high levels of exhaustion markers including PD-1 (**Chapter 4** and **Figure 6),** we theorized that anti-PD-1 would provide durable clinical responses in a significant fraction of MCC patients with advanced disease. Clinical and correlative studies results of the Phase II clinical trial of PD-1 blockade with pembrolizumab in advanced MCC patients are included in **Chapter 6**, where we show that 56% of patients have an objective response to PD-1 as defined by RECIST, version 1.1[52]. Excitingly, both virus-positive and virus-negative patients respond to pembrolizumab. Surprisingly, neither the expression of the ligand for PD-1 (PD-L1) on tumor cells, or the degree of intratumoral CD8+ T cell infiltration, was associated with response to pembrolizumab.

Study of the number, function and localization of MCPyV-specific T cells during immunotherapy may provide mechanistic insights. To understand why we observe robust clinical responses in many but not all patients, we exhaustively phenotyped MCPyV-specific T cells before and after therapy with these agents.

Examples of specific questions that were addressed include:

- How many patients had MCPyV-specific T cells in their PBMC or tumors, as quantified by percent of all CD8 T cells that were MCPyV tetramer+, or presence of T cell reactivities to MCPyV-specific peptides?
- Did the presence of MCPyV-specific T cells correlate with outcome?
- Did the frequency of MCPyV-tetramer + T cells increase after therapy?

- As noted previously, MCPyV-specific T cells can be exhausted *ex vivo*. Does the phenotype of MCPyV-specific T cells (as measured by extracellular markers) change after therapy?
- Does the effector function of MCPyV-specific T cells (as measured by cytokine secretion) increase after therapy?

In addition, insights may be gained by studying the composition of MCPyV-specific TCRβ clonotypes within tumors, including ability to respond to therapy or mechanism of therapeutic action. By sequencing the TCRβ repertoire we gain the ability to understand how diverse the immune infiltrate is, measured by 'clonality' (**Figure 7**). In tumors dominated by a few clonotypes of T cells (ie, antigen-specific T cells), clonality is high, while it is lowest in tumors where T cell clonotypes are all present at exactly the same number. Increased tumor clonality was predictive of patient response to PD-1 immunotherapy in a clinical trial with metastatic melanoma patients[53], and likely signified a clonal dominance of melanoma-specific T cells in the tumor that were poised for re-invigoration by PD-1. We asked this same question in our own trial of PD-1 in MCC, by sequencing all TCRβ clonotypes in pre-treatment tumors from every patient.

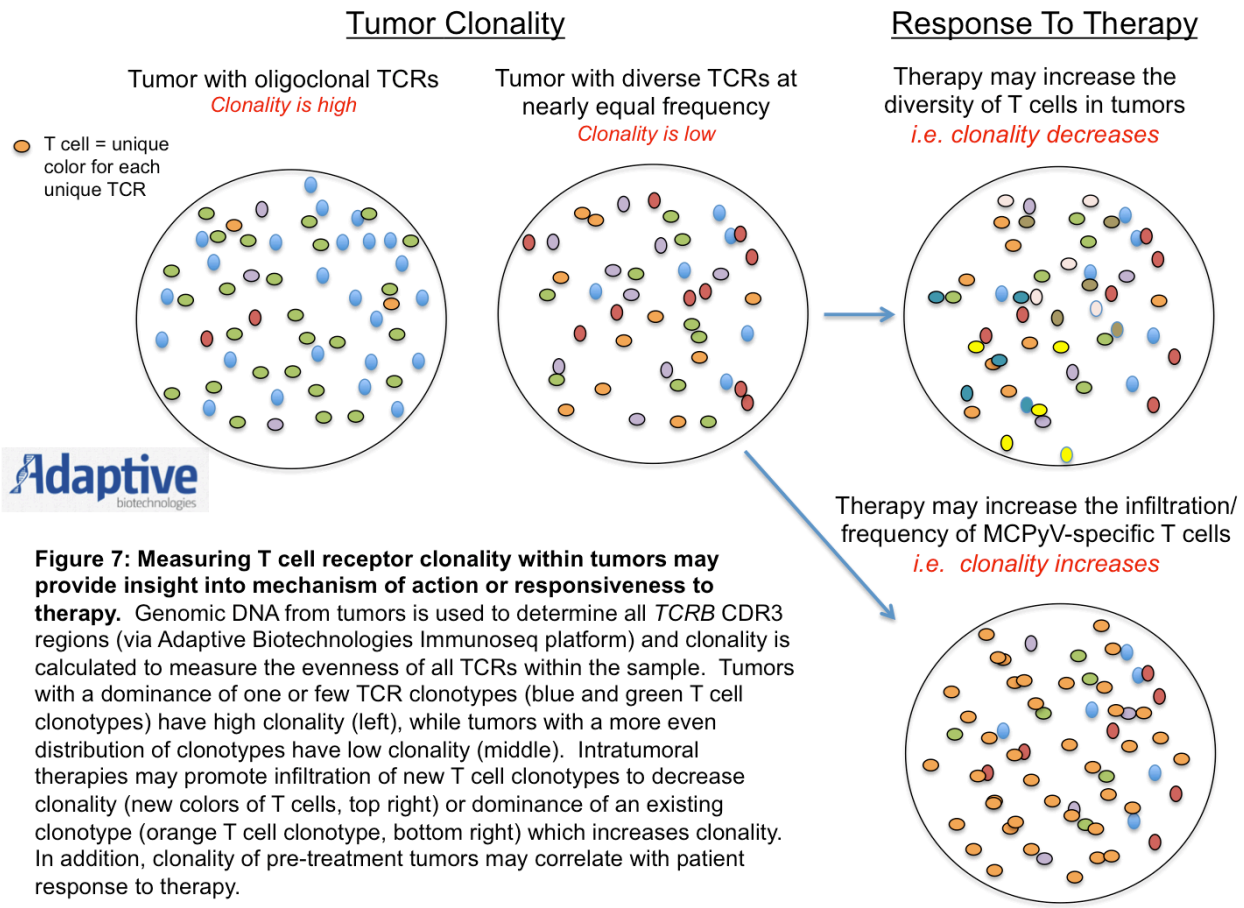

Figure 7: Measuring T cell receptor clonality within tumors may provide insight into mechanism of action or responsiveness to therapy. Genomic DNA from tumors is used to determine all *TCRB* CDR3 regions (via Adaptive Biotechnologies Immunoseq platform) and clonality is calculated to measure the evenness of all TCRs within the sample. Tumors with a dominance of one or few TCR clonotypes (blue and green T cell clonotypes) have high clonality (left), while tumors with a more even distribution of clonotypes have low clonality (middle). Intratumoral therapies may promote infiltration of new T cell clonotypes to decrease clonality (new colors of T cells, top right) or dominance of an existing clonotype (orange T cell clonotype, bottom right) which increases clonality. In addition, clonality of pre-treatment tumors may correlate with patient response to therapy.

In addition, when matched pre- and post-treatment tumors were available, we asked whether tumor clonality increased (indicating an enrichment or proliferation of few MCPyV-specific clonotypes) or decreased (indicating an infiltration of new T cell clonotypes) after treatment with PD-1 or intratumoral therapies. These results may help us to understand the mechanism by which these therapies are effective and augmenting the immune response. Lastly, in a subset of tumors from patients with MCPyV tetramer + T cells, we identified the MCPyV-specific clonotypes within tumors by comparing the intersection of TCRβ clonotypes found in both the tetramer-sorted sample and the tumor. We have tracked these MCPyV-specific T cells in tumors during therapy to more directly elucidate any change in frequency of the MCPyV-specific T cells.

We present these preliminary, unpublished findings in **Chapter 7.** These studies may provide further insight into the mechanism of action of PD-1 blockade in MCC. In addition, it is

likely that a combination of immunotherapies will be necessary for therapeutic efficacy in many patients. Studying the response of T cells to one particular agent may suggest which secondary agent would be particularly beneficial. This study might also unveil biomarkers to predict which patients will or will not respond to a particular therapy.

In summary, our unparalleled access to MCC blood and tumor samples has allowed us to further enhance our understanding of the both the endogenous immune response to MCPyV, as well as how the immune response may be enhanced by various therapeutic interventions. In **Chapters 3-4** we identified two mechanisms by which the immune system may be impaired: 1) extreme diversity of TCRs restricted to one predominant MCPyV epitope, which may mean that some patients lack MCPyV-epitope-specific T cells that are high avidity and/or able to infiltrating tumors and 2) high expression of exhaustion markers that inhibit T cell signaling on MCPyV-specific CD8+ T cells. In addition, our studies in **Chapters 5-7** have not only revealed that new immune-based therapies are effective in recurrent and metastatic MCC, but have contributed to our understanding of the mechanisms of therapeutic action for these treatments. Importantly, these studies will contribute to improving the efficacy of both existing and emerging treatment modalities for advanced stage MCC and have provided hope for patients diagnosed with this aggressive disease.

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Skin Cancer (W Sharfman, Section Editor)

Emerging and Mechanism-Based Therapies for Recurrent or Metastatic Merkel Cell Carcinoma

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Opinion statement

Merkel cell carcinoma (MCC) is a rare but aggressive neuroendocrine skin cancer with a disease-specific mortality of approximately 40 %. The association of MCC with a recently discovered polyomavirus, combined with the increased incidence and mortality of MCC among immunocompromised patients, highlight the importance of the immune system in controlling this cancer. Initial management of MCC is summarized within the NCCN guidelines and in recently published reviews. The high rate of recurrent and metastatic disease progression in MCC, however, presents a major challenge in a cancer that lacks mechanism-based, disease-specific therapies. Traditional treatment approaches have focused on cytotoxic che-

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motherapy that, despite frequent initial efficacy, rarely provides durable responses and has high morbidity among the elderly. In addition, the immunosuppressive nature of chemotherapy is of concern when treating a virus-associated cancer for which survival is unusually tightly linked to immune function. With a median survival of 9.6 months after development of an initial metastasis (n=179, described herein), and no FDA-approved agents for this cancer, there is an urgent need for more effective treatments. We review diverse management options for patients with advanced MCC, with a focus on emerging and mechanism-based therapies, some of which specifically target persistently expressed viral antigens. These treatments include single-dose radiation and novel immunotherapies, some of which are in clinical trials. Due to their encouraging efficacy, low toxicity, and lack of immune suppression, these therapies may offer viable alternatives to traditional cytotoxic chemotherapy.

Introduction

MCC is diagnosed in approximately 1,600 patients each year in the United States [1], a reported incidence that has grown rapidly due to both better pathologic diagnostic tools and an increase in the risk factors associated with this cancer. These factors include age greater than 50 years, Caucasian ethnicity, UV exposure, and immune suppression, although more than 90 % of MCC patients have no known immune dysfunction [2]. Primary MCCs are most frequently found on the head and neck (29 %), followed by lower (24 %) and upper (21 %) extremities [2].

MCC takes its name from the Merkel cell, a part of the somatosensory system located in the basal layer of the epidermis, with which it shares characteristics, such as neuroendocrine granules and cytokeratin-20 expression. MCC is associated with Merkel cell polyomavirus (MCPyV) in approximately 80 % of cases [3]. Although MCPyV is ubiquitous, in MCC the virus has undergone two rare mutations that contribute to unchecked host cell growth. MCPyV large T-antigen binds the retinoblastoma protein, promoting E2F activity and cell cycle progression [4]. Expression of the large T-antigen also increases expression of host cell survivin, an anti-apoptosis oncogene [5]. Less is known about the biology of virus-negative MCCs. These tumors have been associated with activating mutations in *PI3KCA* [6], inactivating mutations in p53 [7•], and poorer MCC-specific survival [8, 9], although this point is controversial [10].

MCC generally presents as a painless nodule that is red, purple, or skin-tone (in cases of deeper presentation). The tumor typically grows rapidly in the span of a few months. In one large study of 5,823 patients, the majority (66 %) presented with localized disease, whereas 27 % had lymph node involvement and 7 % had metastatic disease at presentation [11].

There is no "gold standard" for the diagnosis of MCC, but the triad of MCPyV and cytokeratin-20 (CK20) positivity plus location in a sun-exposed area is diagnostic. CK20 is positive in 88-100 % of MCCs, whereas CK7 and TTF-1, markers of small cell lung cancer, are typically negative [12, 13].

The American Joint Committee on Cancer TNM Staging Classification for MCC should be used for a comprehensive staging reference [11, 14]. Local disease is classified as stage I for tumors ≤2 cm and as stage II for tumors >2 cm, with A or B subclassification based on pathologic versus clinical evaluation of lymph nodes. Regional nodal disease is stage IIIA when nodes are examined by pathology only and stage IIIB when clinically apparent by examination or radiologic study. Stage IV denotes distant metastatic disease. Sentinel lymph node biopsy (SLNB) is a useful staging tool; multiple studies indicate that pathologic examination is a more sensitive method of lymph node evaluation, and in several studies pathologic nodal examination detected microscopic disease in 23-32 % of clinically negative nodes [15–17].

There are no clear data-driven consensus guidelines for how patients should be tracked to detect disease progression early. Clues for detecting subclinical disease progression can be taken from data

relating to initial workup. Several imaging modalities can be used to monitor disease progression. (F-18-FDG)-PET scan is more sensitive than CT for detecting positive lymph nodes (sensitivity of 83 % vs. 47 %) [18] and bone metastases [19]. FDG-PET also was found to be more sensitive than radiolabeled octreotide scintigraphy (^{111}In-Pentetreotide, OctreoScan), which labels somatostatin receptor expressing tumors [20]. However in our experience, PET scans performed without contrast-enhanced diagnostic CT can miss liver metastases, perhaps because of the higher baseline glucose metabolism in the liver.

Serology can be used to detect IgG against the MCPyV T antigen in 40.5 % of MCC cases (n=205). Viral antigen titers track closely with disease burden, decreasing eightfold per year in patients without recurrence and increasing rapidly in patients with progressive disease [21]. A subsequent study is ongoing in our center. This assay continues to perform well, both to reassure patients and to identify clinically occult recurrences. We anticipate routine clinical availability of this T-antigen serology study in 2013 (for details see www.merkelcell.org).

MCC-specific 5-year survival is 63-87 % for patients with local disease and 39-42 % for those with regional nodal disease but only 0-18 % for patients with distant metastatic disease [11, 22]. Among all patients with local or regional disease, two independent studies each found that 48 % of patients ultimately developed recurrent disease. Among patients who recurred, the median time between diagnosis and recurrence was 9 months [15, 22]. In a cohort of 179 MCC patients who developed distant metastatic disease, the median survival from the time of initial metastasis was approximately 9.6 months (289 days; Fig. 1). Some markers of improved outcome appear to have very strong support, including multivariate analysis in multiple larger studies. These parameters include lower-stage disease [11, 23], no chronic immunosuppression [24, 25], intratumoral infiltration by CD3 [26] (or CD8 [27]) T cells, and absence of lymphovascular invasion [23] [our unpublished data]. Other parameters are supported by multivariate analysis in a single study. These include a nodular (versus infiltrative) growth pattern [23], the absence of p63 expression [28], and increased titer of anti-

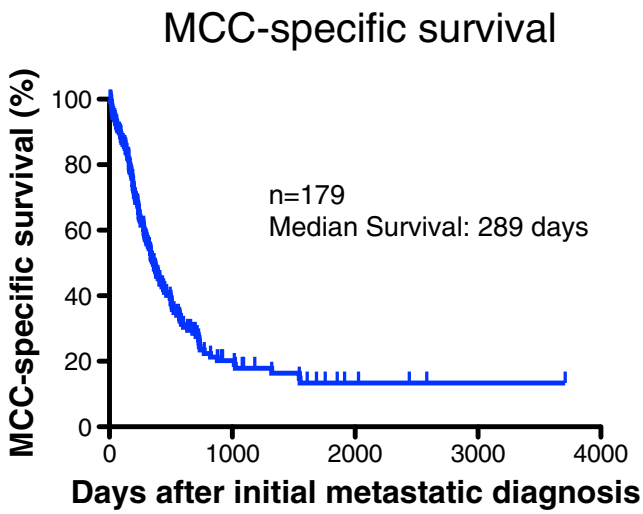

Figure 1. Disease-specific survival in patients who developed metastatic Merkel cell carcinoma. Survival data are shown from 179 patients with metastatic MCC who were followed through the University of Washington/Fred Hutchinson Cancer Research Center. Median survival was 289 days from initial diagnosis of metastatic disease. When measured starting at the time of developing metastatic disease, there were no significant differences in survival based on the initial stage at presentation (data not shown). However, stage greatly influenced the likelihood of and median time to developing metastatic disease [11]. Among patients who developed metastatic disease, the interval between initial diagnosis and metastasis was longer for patients presenting with less advanced stage. Overall survival is very similar to the MCC-specific survival curve shown above.

bodies to the Merkel polyomavirus capsid protein (VP1) [25]. Another set of outcome measures remains controversial because conflicting data exist. These include a better prognosis associated with decreased Breslow tumor thickness [23, 29] and positive Merkel polyomavirus status [8–10].

Treatment

Searches of the FDA website and CenterWatch.com for all available years (1995-2012) yielded no FDA-approved agents for this cancer. A summary of the following therapeutics can be found in Table 1.

Surgery

In most cases of recurrent or metastatic disease, surgical management does not have a significant role due to the high probability of subclinical microscopic disease. One situation where surgery has utility is in the case of free tissue transfer reconstruction after microvascular anastomosis [30]. This approach has the advantage of allowing additional radiation therapy to anatomic areas that previously underwent significant irradiation.

Pharmacologic

MCC is generally considered to be a chemotherapy-sensitive tumor [31] with tumor regression observed in the majority of cases treated with first-line chemotherapy. Whereas cytotoxic chemotherapy is the dominant mode of treatment for advanced disease, it is virtually never curative and is associated with significant toxicity. Side effects, such as myelosuppression (including neutropenic fever), nausea/vomiting, fatigue, and hair loss are common, with therapy-related death occurring in up to 16 % of older patients [32]. Chemotherapy regimens for MCC are mostly extrapolated from those used for small cell lung cancer (SCLC), another neuroendocrine tumor. The commonly used regimens are described below:

Chemotherapy for Metastatic Disease

The data on cytotoxic chemotherapy for MCC is mostly obtained from retrospective institutional reviews or meta-analysis of small case series, and hence is subject to reporting bias. In a review of 31 patients with local recurrences, 68 % responded to first line chemotherapy (regimen unspecified), whereas 59 % of 103 patients with distant disease demonstrated responses [33]. In a retrospective study, 69 % of patients with locally advanced disease and 57 % with metastatic disease responded to first-line chemotherapy. However, survival was limited to an average of 24 or 9 months with locally advanced or metastatic disease, respectively. Death from drug toxicity was high (7.7 %) in this disease that mostly affects older patients [32].

Available data for specific chemotherapy regimens, described below, unfortunately represent a mixture of both local and advanced disease. Because of this limitation, and the fact that some patients received radiation in addition, the reported response rate is likely overly optimistic for chemotherapy alone in the setting of recurrent and metastatic disease.

Table 1. Summary of therapies discussed, including mechanism of action and evaluation of evidence for use in MCC

Treatment	Target cell	Mechanism	Strength of data
Traditional chemotherapy agents			
Cisplatin/carboplatin	Tumor	Crosslinks DNA	NCCN [32, 33]
Etoposide	Tumor	Inhibits topoisomerase II	NCCN (IV) [32, 33], CR(4) (oral) [36]
Cyclophosphamide	Tumor	DNA alkylating agent	NCCN [32, 33]
Doxorubicin	Tumor	DNA intercalator	NCCN [32, 33]
Vincristine	Tumor	Inhibits microtubule assembly	NCCN [32, 33]
Topotecan	Tumor	Inhibits topoisomerase I	NCCN
Interventional procedures			
Fractionated radiation	Tumor	DNA damage	NCCN
Single dose radiation	Tumor	DNA damage, immune stimulation (?)	CR(6) [42]
Mechanism-based drugs			
Octreotide	Tumor, endothelium (?)	Antiproliferative, vasoconstriction and tumor necrosis (?)	CR(2) [47, 48] Ph-I in preparation [50]
^{177}Lutetium-octreotide	See above	Coupled radio-peptide; see above plus DNA damage	CR(2) [51, 52] Ph-II ongoing [53]
Pazopanib	Tumor, endothelium	Antiproliferative and inhibits angiogenesis	CR(1) [55]
PI3K inhibitors	Tumor	Antiproliferative	MCC *in vitro* [6, 56] Ph-II other CA ongoing
Lorvotuzumab mertansine (IMGN901)	Tumor	Inhibits microtubule assembly in CD56 expressing cells	Ph-I [58]
YM-155	Tumor	May downregulate survivin to promote apoptosis	MCC xenograft/*in vitro* [5], Ph-II other CA
Immunotherapy			
IL-12 DNA electroporation	Lymphocytes, NK cells	Promotes Th1 response; increases IFN-γ and cytolytic activity	Ph-I/II ongoing [71]
Interferon (intralesional)	Tumor	MHC-I upregulation, antiproliferative, antiangiogenic	CR(4) [72, 75], *in vitro* [73, 74]
Anti-PD-1	CD8+ T cell	Blocks inhibitory/exhaustion signaling to CD8+ T cells	Ph-I other CA [60]
Anti-PD-L1	Tumor, APC	Blocks inhibitory/exhaustion signaling to CD8+ T cells	Ph-I other CA [61]
Ipilimumab	CD8+ T cell	Blocks CTLA-4 mediated inhibition of immune activation	Ph-III other CA [63, 64]
4-1BB (CD137) agonist	CD8+ T cell	Costimulatory signal for immune activation	Ph-I ongoing [67], xenografts other CA [66]
Transgenic T cell receptors (TCRs)	Tumor	Engineered tumor-antigen targeted T cells	Ph-II other CA [78]
STAR conjugates	Tumor	Drug delivery to tumor	Ph-I other CA [79]
Adoptive T cell therapy	Tumor	Expansion of and restoration of cytotoxic capability to tumor-targeted lymphocytes	Ph-I/II in preparation [77]
Agents lacking significant efficacy			
Interferon (systemic)	Tumor	See above, no evidence for systemic efficacy	CR(4) [83, 84]
Imatinib	Tumor	Antiproliferative: blocks KIT signaling	Ph-II [80]
Oblimersen (Genasense)	Tumor	Downregulates Bcl-2 to promote apoptosis	Ph-II [82]

Under each subheading, therapies are listed in order of frequency of use/strength of data
NCCN, "appropriate" therapy per NCCN Consensus Guidelines for Merkel Cell Carcinoma [35]; CR, case report (number of patients reported); Ph-I, Phase I clinical trial; Ph-II, Phase II; Ph-III, Phase III; CA, cancer; NK, natural killer cell; APC, antigen-presenting cell

Etoposide + Platinum Agent (Cisplatin or Carboplatin)

Platinum plus etoposide (PE) is the most commonly used chemotherapy regimen for MCC. For local and advanced disease, this combination gave an overall response rate of 60 % [33]. In a study of radiation plus PE in a mixture of local and advanced patients, there was 76 % 3-year survival rate [34].

Cyclophosphamide, Doxorubicin (or Epirubicin), and Vincristine (CAV)

CAV is another chemotherapy combination commonly used in MCC. A retrospective review of local and advanced MCC cases treated with CAV found an overall response rate of 76 %, with significant toxicities including death in 3.5 % of patients [33].

Topotecan

This topoisomerase I inhibitor is commonly used for small cell lung cancer and can be considered for use in older patients [35].

Oral Etoposide

In a small recent case series, oral etoposide led to durable remission or stable disease in four patients, with minimal side effects, including neutropenia in one patient [36].

Adjuvant Chemotherapy

Current data on adjuvant chemotherapy are insufficient to determine its potential usefulness. In the single largest study of 76 patients with nodal disease, there was a trend toward poorer 4-year survival in those who received adjuvant chemotherapy (42 %, n=23) compared with those who did not (60 %, n=53) [15]. Although this was not a randomized trial and comorbidities may have played a role, this certainly does not suggest a clinically meaningful benefit of adjuvant chemotherapy. It is plausible that the potential cytotoxic benefits of adjuvant chemotherapy may be offset by chemotherapy-induced immunosuppression in this immune-sensitive malignancy. The toxicity considerations in a mostly elderly population also are important when discussing the role of adjuvant chemotherapy with patients.

Interventional Procedures

Radiotherapy – General Principles

MCC has long been known to be a radiosensitive tumor [37], and radiotherapy plays an integral role in the treatment of every stage of this cancer. In the curative setting, it is used commonly in combination with surgical excision or as monotherapy when surgery cannot be performed or the morbidity of surgery is prohibitive. Optimal local treatment of MCC requires radiotherapy after a complete surgical resection. Surgery by itself is inadequate treatment in all but highly selected cases of very early

stage/favorable tumors (for example, <1 cm primary, without lymphovascular invasion, and sentinel lymph node negative in a nonimmunesuppressed patient). Adjuvant radiation has been associated with improved overall survival from 45 to 63 months in a study by Mojica et al., with benefit especially noted for tumors >2 cm [38]. In another retrospective meta-analysis of 1,254 patients, adjuvant radiation was associated with a disease-specific survival benefit (hazard ratio, 0.62) compared with surgery alone [39].

Radiation monotherapy is a highly successful strategy for the treatment of MCC in our experience with local control rates exceeding 90 % [40]. The radiation dose for curative intent is 60-66 Gy to the tumor mass during monotherapy and 50 Gy when addressing residual microscopic disease. This is given at a standard fractionation of 2 Gy per fraction for 25-33 treatments over 5-6.5 weeks [35].

Radiotherapy is an effective modality in the palliative setting of incurable metastatic or recurrent MCC. It has typically been delivered in multiple fractions (5-20) and reliably provides relief from cancer symptoms with minimal side effects, thus improving the quality of life of patients. The responses to target lesions are generally durable.

Single-Dose Radiotherapy

The effects of radiation on the immune system are not fully understood. It was recently found that a single fraction of high-dose radiation stimulates lymph node priming as well as CD8 T-cell–mediated reduction of primary and metastatic tumors in a mouse model [41••]. Subsequent doses of fractionated radiation can suppress the activity of recruited lymphocytes, thus single-dose treatment may have advantages in terms of promoting immune function. In addition, RT given in a single, large-dose fraction of 8 Gy is well known to provide safe and effective palliation for bone metastases and a single fraction is logistically very convenient for patients. At our center, we treated 15 MCC tumors with a single fraction of 8-Gy radiation during a 1-year period. These included 7 chemorefractory tumors. There were 11 complete and 4 partial (>50 %) responses. No side effects were reported during a median follow-up of 5 months [42]. Although these studies are in the early stages, a single fraction of 8-Gy radiotherapy may offer a better therapeutic ratio compared with traditional treatments for MCC. In addition to its superb side-effect profile, this approach is cost effective and convenient for patients who are ill with metastatic disease for whom multiple visits to a radiotherapy center are a major burden.

Brachytherapy

Brachytherapy is the precise delivery of short-range radiation (within a few millimeters) by positioning the radioactive source within or in close proximity to the tumor, and is derived from the Greek *brachy*, meaning close. Its use is limited but can be effective in widely disseminated cutaneous disease. A case report showed a durable response of multiple cutaneous metastatic

MCC nodules, including one untreated lesion, in the right lower extremity after delivery of 12 Gy by brachytherapy [43].

Palliation

The goal of palliative care is to provide pain control and support to patients with serious illness. In a recent randomized study of lung cancer patients, early palliative care led to significant improvements in both quality of life and mood. Unexpectedly, despite receiving less aggressive care at the end of life, patients randomized to palliative care survived longer than those who received standard care alone [44]. It is ideal to discuss palliative care options early in the treatment process for patients with high risk or advanced disease. The ASCO Palliative Care Checklist [45] and the NCCN Palliative Care Guidelines [46] provide useful tools for these discussions.

Mechanism-Based Therapies

Octreotide

Octreotide is a potent, biologically stable octapeptide analog of the naturally occurring hormone somatostatin. Somatostatin has an antiproliferative effect on neuroendocrine tumor cells and may inhibit tumor angiogenesis. mRNA expression of somatostatin receptor 2 has been demonstrated on 90 % of MCCs [47], providing rationale for treatment of MCC with this class of drugs. Imaging via radiolabeled octreotide (OctreoScan) can be a useful clinical indicator of physiologic octreotide binding to a given patient's tumor. Among two reported cases, encouraging responses were seen in both patients [48, 49]. A phase I clinical trial in MCC with pasireotide, another somatostatin analog, is upcoming [50]. Two case reports in which a therapeutic radioisotope was coupled to a peptide of this class demonstrated clinical benefit with a favorable safety profile [51, 52]. A Phase II clinical trial with such an agent is currently underway in neuroendocrine cancers, including MCC [53].

Pazopanib

Pazopanib is a receptor tyrosine kinase inhibitor that targets VEGFR-1, -2, -3, PDGFR-α, -β, and c-kit. Immunohistochemistry has detected VEGF-A, VEGF-C, VEGF-R2, and PDGF-α expression in 72-91 % of MCCs [54]. Pazopanib is hypothesized to inhibit both tumor growth and angiogenesis and is currently FDA-approved for the treatment of renal cell carcinoma and soft tissue sarcoma. The drug is generally well tolerated and is not considered to be immunosuppressive. In a recent case report of oral pazopanib used to treat a patient who had failed multiple prior treatment modalities, the patient's scalp tumor completely resolved after 2 months of pazopanib with a partial response in her pulmonary metastases that lasted 6 months [55•]. The investigators found a germline mutation in the gene for PDGFR-α in three patients, suggesting a possible role of the gene in predisposition toward MCC or as a marker for potential treatment response.

PI3K Inhibition

A recent study identified activating PI3KCA gene mutations in 10 % of MCCs analyzed, with the majority found in virus-negative cancers [6]. MCC cell lines are sensitive to PI3K inhibitors currently in clinical development [6, 56].

YM-155

Survivin is a cellular protein with antiapoptotic properties that is commonly upregulated in MCC. Its expression tracks with levels of Merkel polyomavirus large T antigen. YM-155 is a small molecule that has been suggested to downregulate survivin. This drug causes cell death in MCC cell lines *in vitro* and appears to be cytostatic in a mouse xenograft tumor model [5]. A previous Phase 1 trial of YM-155 in other cancers showed that it can be administered safely and is well tolerated [57].

Lorvotuzumab Mertansine (IMGN901)

IMGN901 is an antibody-drug conjugate consisting of a maytansinoid microtubule assembly inhibitor coupled with a humanized monoclonal antibody to CD56, which is expressed on nearly all MCCs. In a phase 1 trial that included 12 MCC patients, two patients experienced durable complete responses after treatment with IMGN901 given IV at either 36 or 60 mg/m^2/day [58].

Emerging and Viral Antigen-Directed Immunotherapies

The three immune-stimulatory antibodies below are all being actively investigated in clinical studies for various cancers and may have benefit in MCC. Although there are no MCC-specific clinical trials ongoing for these agents, the hope is that they will be forthcoming.

PD-1/PD-L1 Inhibitors

PD-1 is an inhibitory cell surface receptor that blocks T-cell receptor (TCR) signaling on lymphocytes. Persistent, unresolved viral infections often are associated with functionally impaired T cells that have increased expression of surface PD-1 [59]. A Phase I trial of a PD-1 inhibitor demonstrated cumulative response rates of 18 % in non-small cell lung cancer patients, 28 % in melanoma patients, and 27 % in renal-cell cancer patients. Importantly, 68 % of responses were durable for at least 1 year. Response to PD-1 blockers was strongly linked to tumors expressing PD-L1 [60•]. Antibodies blocking PD-L1 also showed durable tumor regression in a recent Phase I trial with responses of 6-17 % in the same three cancers [61]. Due to the viral etiology and immunosuppression associated with MCC, it is very possible that PD-1 blockers would have efficacy in this cancer. It was recently found that PD-1 is upregulated on Merkel polyomavirus-specific CD8 T cells compared with control virus-specific cells in MCC patients [62].

Ipilimumab

Ipilimumab is a monoclonal antibody that blocks the inhibitory receptor CTLA-4, increasing T-cell activation. It has been shown to improve survival of metastatic melanoma patients [63, 64].

A case report of ipilimumab combined with radiotherapy to treat metastatic melanoma demonstrated an abscopal effect of tumor shrinkage in untreated lesions, as well as increased antibody titers to diverse melanoma antigens [65]. This may be relevant to MCC, especially with the excellent

tolerability of single-dose radiation treatment that could be combined with systemic immunotherapies.

4-1BB (CD137) Agonist

4-1BB is a TNF-family costimulatory receptor expressed on activated T cells. In preclinical trials, antibodies that bind this receptor increase NF-κB activity leading to cytokine production, increased leukocyte proliferation, and reduced tumor growth [66]. A phase I trial is currently underway in patients with advanced/metastatic solid tumors [67]. We have found that Merkel polyomavirus-specific T cells express higher levels of CD137 compared with control virus-specific cells, suggesting a role for 4-1BB agonists in treating MCC [62].

Interleukin-12 DNA Electroporation

IL-12 is a Th1 skewing cytokine that induces proliferation, cytotoxicity and IFN-γ production by preactivated natural killer and T cells. Systemic administration of rIL-12 has been limited by toxicity and temporary immune suppression [68], promoting investigation into local administration routes. In a mouse melanoma model, intratumoral injection of a plasmid encoding IL-12 followed by electroporation caused several desirable immune effects. These included IL-12 and IFN-γ induction, enhanced lymphocyte migration, reduced tumor vascularity, and tumor elimination in 47 % of treated mice [69]. A Phase 1 study of electroporated IL-12 in patients with metastatic melanoma demonstrated complete resolution of distant, nonelectroporated lesions in 10 % of patients, with partial or stable response in 42 % of patients and minimal systemic side effects [70]. A phase II trial is currently ongoing for MCC [71].

Intralesional Interferon

Whereas MCC is associated with immune suppression, more than 90 % of patients are not immunocompromised and these tumors have thus likely acquired immune evasion mechanisms to avoid detection by cytotoxic T cells. Indeed, more than half (51 %) of 114 MCC tumors demonstrated downregulation of MHC-I, an established mechanism for CD8 T-cell evasion. *In vitro*, interferon treatment of MCC cell lines led to reversal of this MHC-I downregulation [72] and induced apoptosis [73, 74]. In a case report, daily IFN-β injections into a patient's forearm metastases resulted in a durable (>8 years) complete response following 5 weeks of monotherapy [75]. In our pilot studies, intralesional interferon-β (3 MIU, 3× week, for 1-4 weeks) led to increased expression of MHC-I, increased CD8 T-cell infiltration, and local tumor regression among three patients with available pre- and post-treatment biopsy materials [72].

Adoptive T-Cell Therapy

This process involves the enrichment and reinfusion of autologous antitumor T cells into cancer patients. Adoptive cell transfer into metastatic melanoma patients who had been heavily pretreated with lymphodepletion and/or radiation had an objective response rate of 56 % [76]. Although not widely available,

this response rate is superior to other available chemo- and immunotherapies. The persistent expression of non-self (Merkel polyomavirus) antigens in most MCC tumors makes adoptive T cell therapy for this cancer particularly attractive. At our center, we have treated one patient who developed metastatic MCC using Merkel polyomavirus-specific T cells [77] and plan to start a Phase I/II trial that will enroll 16 advanced-stage patients.

Transgenic T-Cell Receptor-Based Therapies

Lymphocytes can be genetically engineered to express transgenic T-cell receptors (TCRs) that recognize cancer antigens. Among 36 metastatic melanoma patients, autologous lymphocytes expressing transgenic TCRs targeting two melanocyte antigens persisted *in vivo* and elicited objective response rates of 19 % or 30 %. However, reactivity toward normal tissues led to significant side effects, such as vitiligo, uveitis, or hearing loss, in more than half of patients [78].

STAR Reagents

Soluble T-cell antigen receptor (STAR) reagents are synthesized TCRs that recognize cancer antigens and are coupled to therapeutic agents including cytokines or radioisotopes. A p53 targeted, HLA-A0201 restricted STAR reagent coupled to IL-2 was found to increase serum IFN-γ in a phase 1 trial in metastatic cancer patients. In this study, 10 of 26 subjects had stable disease after 11 weeks, with one complete remission and minimal toxicity [79]. Advantages of STAR reagents include the potential for commercial viability and "off the shelf" use, although they do need to be compatible with the patient's HLA-type.

Agents Without Apparent Clinical Efficacy

Imatinib is a tyrosine kinase inhibitor with activity against KIT, a receptor tyrosine kinase commonly expressed in MCCs. Imatinib was therefore investigated in a phase II trial with 23 MCC patients. Unfortunately, most patients showed rapid disease progression during treatment [80]. It was subsequently shown that KIT expression in MCC is less common than previously reported and activating KIT mutations are infrequent, perhaps explaining the low efficacy of imatinib in this cancer [81].

Oblimersen (Genasense) is an oligonucleotide that targets and downregulates Bcl-2 expression, increasing apoptosis. In a phase II trial with 12 advanced MCC patients, no objective responses were seen [82].

Systemic interferon has been investigated in MCC. In four metastatic MCC patients treated with systemic interferon-α, no tumor responses were seen [83, 84]. This may have been due to inadequate levels of interferon within the tumor microenvironment or compensatory systemic immune regulation.

Conclusions

While the prognosis for distant metastatic MCC is grim, recent advances in our understanding of cancer immunity have provided rational mechanism-based therapies that are entering clinical testing. The strong links between

the immune system and this virus-associated malignancy provide exciting opportunities for immunotherapy, including the possibility of combining antibody-based therapeutics that stimulate the immune system globally with tumor antigen targeted treatments. Because of fundamental biological differences, virus-negative MCCs may require independent, focused attention to create effective therapeutic approaches.

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Conflict of Interest

No potential conflicts of interest relevant to this article were reported.

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- • Of importance
- •• Of major importance

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Tumor-infiltrating Merkel polyomavirus-specific T cells are diverse & predict improved survival of Merkel cell carcinoma patients

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TRANSLATIONAL RELEVANCE:

Merkel cell carcinoma (MCC) is an aggressive skin cancer without durable treatments for advanced disease. MCC patients can develop CD8+ T cells specific for the oncogenic T-antigens of the Merkel cell polyomavirus (MCPyV). Tumor-infiltrating CD8+ T cells occur rarely but are associated with 100% survival. Adoptive T cell therapy of MCPyV-specific CD8+ T cells has been associated with limited efficacy and requires patients to have autologous MCPyV-specific T cells.

Here, we characterize the T cell receptors (TCRs) of MCPyV-specific T cells recognizing one prevalent MCPyV epitope from 12 patients. We found significant genetic and functional diversity among these TCRs. Patients with a higher frequency of epitope-specific T cells in their tumors had improved MCC-specific survival. T cell clones with greater functionally avidity were isolated from patients with improved MCC-specific outcomes. The high-avidity TCRs pave the way for MCPyV-specific TCR gene therapy.

ABSTRACT:

Purpose: Tumor-infiltrating CD8+ T cells are associated with improved survival of patients with Merkel cell carcinoma (MCC), an aggressive skin cancer causally linked to Merkel cell polyomavirus (MCPyV). Only 4-18% of MCC patients have such CD8+ T cell infiltration. We characterized the T cell receptor (TCR) repertoire restricted to one prominent epitope of MCPyV (KLLEIAPNC, "KLL") and assessed whether TCR diversity, tumor infiltration or T cell avidity correlated with clinical outcome.

Experimental Design: HLA-A*02:01/KLL tetramer+ CD8+ T cells from MCC patient peripheral blood mononuclear cells (PBMC) and tumor infiltrating lymphocytes (TIL) were isolated via flow cytometry. TCRβ (*TRB*) sequencing was performed on tetramer+ cells from PBMC or TIL (*n*=14) and matched tumors (*n*=12). Functional avidity of T cell clones was determined by IFN-γ production.

Results: We identified KLL tetramer+ T cells in 14% of PBMC and 21% of TIL from MCC patients. *TRB* repertoires were diverse (mean of 12 and 29 clonotypes/patient in PBMC and TIL, respectively) and mostly private. An increased fraction of KLL-specific TIL (>1.9%) was associated with significantly increased MCC-specific survival ($p=0.0009$). Forty-two distinct KLL-specific TCRα/β pairs were identified. T cell clones from patients with improved MCC-specific outcomes were more avid ($p<0.05$) and recognized an HLA-appropriate MCC cell line.

Conclusion: T cells specific for a single MCPyV epitope display marked TCR diversity within and between patients. Intratumoral infiltration by MCPyV-specific T cells is associated with significantly improved MCC-specific survival suggesting that augmenting the number or avidity of virus-specific T cells may have therapeutic benefit.

INTRODUCTION:

Merkel cell carcinoma (MCC) is a highly aggressive skin cancer associated with UV exposure, advanced age, immune suppression, and in approximately 80% of cases the Merkel cell polyomavirus (MCPyV)[1-3]. MCC incidence is approximately 2000 cases each year in the US[4-5]. There are no effective, durable treatments for advanced disease, leading to a 5-year survival rate of 0-18% for patients with distant metastatic disease and a median survival of only 9.6 months from diagnosis of initial metastasis to death[5-7]. While approximately half of patients initially respond to chemotherapy, responses are not durable with a median time to progression of only 94 days[8], highlighting the need for improved therapies to treat MCC.

MCPyV is a prevalent, chronic virus that normally does not cause disease but upon rare occurrences of clonal chromosomal integration into the host DNA paired with UV-induced mutations, can cause MCC[1,9-10]. Virus-positive MCC is characterized by persistent expression of the small and truncated large T-Antigens[10], which share a common N-terminus (common T-Ag). Multiple studies have linked the immune system with the incidence and prognosis of MCC. While <10% of MCC patients have systemic immune suppression, these patients have significantly poorer MCC-specific survival[11,12]. The adaptive immune system can recognize and mount protective responses to MCPyV. Specifically, increased intratumoral CD3+ cell counts are an independent prognostic factor of increased MCC-specific survival[13]. Robust intratumoral CD8+ lymphocyte infiltration, though present in <20% of MCCs, has been associated with 100% MCC-specific survival, independent of tumor stage at diagnosis[14,15]. Clinical trials of T cell-activating therapies such as PD-1 axis blockade have shown impressive initial response rates and durability[16], which provide further impetus for the study of T cell-

based therapies for MCC.

CD8+ T cells recognizing at least 17 unique epitopes of the persistently expressed T-antigens of MCPyV can be isolated and tracked in blood and tumors from MCC patients using HLA-I tetramers[17-19] (our unpublished observations). MCPyV-specific CD8+ T cells have been harnessed for adoptive T cell therapy (ACT) and yet resulted in a durable response in just one of four patients treated[20,21]. One strategy to increase the efficacy of ACT, and/or offer T cell-based therapies to patients who lack endogenous MCPyV-specific T cells, would be to engineer T cells to express highly avid MCPyV-specific T cell receptors (TCRs).

To better characterize the MCPyV-specific CD8+ TCR repertoire and measure correlations between TCR clonotype repertoire, intratumoral infiltration, and patient outcomes, we studied primary CD8+ T cells that recognize the epitope KLLEIAPNC (derived from the MCPyV common T-Ag) restricted to human leukocyte antigen (HLA) A*02:01, an HLA class I type present in ~50% of our patient cohort (hereafter, KLL-specific T cells). In parallel to other infections and malignancies[22-26], we hypothesized that diversity or functional avidity of the TCR repertoire recognizing this epitope may be correlated with the effectiveness of the T cell response to MCC *in vivo.*

Using next-generation TCRβ sequencing, we found significant genetic diversity among TCRs recognizing the KLL epitope. Using paired blood and tumor specimens, we can now extend previous findings of positive correlations between MCC tumor T cell infiltration and effector gene signatures[14] to the level of tumor antigen-specific CD8+ T cells. In addition, patients with greater KLL-specific clonotype diversity in their tumors have significantly improved MCC-specific and recurrence-free survival. We studied the

functional avidity of CD8+ clones expressing unique KLL-specific TCRs, and found that clones generally expressed a narrow range of functional avidities that were largely similar within each patient. Only 5 of 28 clonotypes tested from one of four patients recognized a MCPyV+ MCC cell line. This correlated with high functional avidity. These studies have elucidated the genetic diversity of CD8+ T cells restricted to KLL and support continued investigation of methods to increase intratumoral CD8+ T cell infiltration. The avid T cell clones we identified could lend their effector function through transgenic TCR for the ~80% of HLA-A*02+ MCC patients who lack endogenous KLL-specific T cells.

METHODS:

Human subjects and samples: This study was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board and conducted according to Declaration of Helsinki principles. Informed consent was received from all participants. Subjects were HLA class I typed via polymerase chain reaction (PCR) at Bloodworks Northwest (Seattle, WA). All samples were clinically annotated with long-term patient follow-up available. PBMC**:** Heparinized blood was obtained from MCC patients and peripheral blood mononuclear cells (PBMCs) were cryopreserved after routine Ficoll preparation at a dedicated specimen processing facility at FHCRC. Patient Tumors: Fresh MCC tumor material from core and/or punch biopsy samples were processed and TIL cultured for two weeks before analysis as described[17]. For excised tumors of larger volume (>1 cm^3), the remaining tissue was digested as described[18] and single cell suspensions were cryopreserved.

T cell receptor β sequencing and analysis: Tetramer+ Cells: At least 2 million PBMC or TIL were stained with anti–CD8-PE antibody (Clone 3B5, Life Technologies), A*02/KLL-APC tetramer (Immune Monitoring Lab at FHCRC) and 7-AAD viability dye (BioLegend). Cells in the tetramer+, $CD8^{high}$ region were sorted via FACSAriaII (BD) and flash frozen (average of 710 cells from PBMC (n=9), 5776 cells from TIL (n=5), range 350-8,000 and 1844-12799, respectively). Samples were submitted to Adaptive Biotechnologies (Seattle, WA) for genomic DNA extraction, *TRB* sequencing and normalization. All *TRB* sequences detected in ≥2 cells (estimated number of genomes ≥2) were categorized as tetramer+ clonotypes. Whole tumor sequencing: Primary

tumors were used for analysis, except when patients presented with unknown primaries and nodal disease (n=2) or metastatic disease (n=1), or when primary tumor tissue was limited and tumor from lymph at time of presentation was also analyzed (n=1). Tumor samples consisted of molecular curls of 25 microns from formalin-fixed, paraffin embedded (FFPE) tissue blocks (n=10), nodal tumor digest frozen *ex vivo* (n=1) or flash frozen core biopsy of a metastatic lesion (n=1). Samples were submitted to Adaptive Biotechnologies as described above. Tetramer+ cell infiltration: KLL-specific clonotypes within tumors (n=12 tumors) were identified based on TCRβ CDR3 amino acid sequences from the tetramer-sorted samples. The frequency of all KLL-specific T cells within each tumor is reported as the cumulative percentage of productive sequencing reads of clonotypes detected in both the tetramer-sorted sample and the tumor.

Survival and recurrence analysis: Statistical analyses were performed on Stata software version 14.0 for Macintosh (StataCorp, College Station, TX) and Prism 6 for Mac OS X (Graph Pad Software, Inc). MCC-specific survival is defined as the interval from the diagnostic biopsy date to death by MCC. Recurrence-free survival defined as the interval from the diagnostic biopsy date to the date of a MCC recurrence, last follow up or death by MCC. Log-rank analysis was performed and a p-value of .05 was considered to be statistically significant. Kaplan-Meier survival curves were created to visualize MCC-specific survival and recurrence-free survival data; groupings of patients were based on percentage of tetramer+ T cells in the tumor (Higher = 1.9-18%, n=9 versus Lower = 0-0.14%, n=2) as well as number of T cell clonotypes (Many = 5-108, n=7; versus Few = 0-3, n=4) were selected *a priori*. Patients who were alive at the last

time of follow-up were censored on their last day of follow-up and patients who died of unknown causes were censored on their day of death.

Creation of KLL-specific T cell clones: PBMC or lymphocytes from tumor digest were stained and sorted as described above into T cell medium (TCM) containing RPMI, 8% human serum, 200nM L-glutamine and 100 U/ml Penicillin-Streptomycin, and cloned at 0.25 to 3 cells per well with allogeneic irradiated feeders, IL-2 (Hemagen Diagnostics) and PHA (Remel) as described[29] with addition of 20 ng/mL rIL-15 (R&D Systems) after day 2. After 2 weeks, microcultures with visible growth were screened for specificity via tetramer; TCR variable beta chain (TCRVβ) expression was assessed by staining clones with fluorescent anti-TCRVβ antibodies (IOTest Beta Mark, Beckman Coulter). Wells selected for screening, expansion, and TCR analysis came from plates with <37% of cultures having visual growth, yielding a 95% chance of clonality per the Poisson distribution[30]. Cultures with tetramer+ cells, reactivity to peptide and dissimilar TCRVβ chains were further expanded with IL-2 and anti-CD3 mAb (Miltenyi Biotec) as described[17], plus 20 ng/mL rIL-15. Prior to harvesting RNA for TCR analysis, cultures were held at least 2 weeks to minimize persistent feeder cell-derived RNA. CD8-independent Tetramer Staining**:** Clones were stained with a HLA-A*02:01/KLL tetramer containing D227K/T228A mutations in HLA-A*02, using methods as above. These mutations abrogate HLA class I:CD8 binding to identify clones expressing TCRs with the ability to bind independent of CD8 stabilization, indicating high TCR avidity[31,32].

TCR α & β sequencing of clones: Simultaneous sequencing of TCRα and TCRβ

repertoires was performed as described[33]. Briefly, total RNA was isolated from clonally expanded populations using Qiagen RNeasy Plus, followed by One Step RT/PCR (Qiagen) primed with multiplexed TCR primers. This reaction was used as template with a second set of nested TCRα and TCRβ primers, followed by PCR to allow the addition of barcoding and paired end primers. Templates were purified using AMPure (Agencourt Biosciences) then normalized prior to running on Illumina MiSeq v2-300. Pairs of 150 nucleotide sequences were merged into contigs using PandaSeq[34]. Merged sequences were then separated according to inline barcodes identifying the plate and well of origin, generating one file of derived sequences for each clone of interest. Files for each clone were processed with MiXCR[35] to identify and quantify clonotypes and assign VDJ allele usage.

T cell functional assays: T cell clones were tested for specificity and functional avidity via cytokine release assays. Cytokine Release with Peptide-pulsed Targets**:** Secreted IFN-γ was measured after co-incubating $2x10^4$ clonal KLL-specific T cells with $5x10^4$ T2 cells plus antigenic peptide at $\log_{10}$ dilutions to final concentration from 10^{-6} to 10^{-12} molar in 200 μl TCM for 36 hours. Due to possible oxidation and dimerization of cysteine residues in the antigenic KLLEIAPNC peptide, the homolog KLLEIAPNA was used to allow for efficient HLA class I presentation; similar substitution has been shown to not alter recognition of HLA-peptide complex by TCRs raised against the native peptide[36]. IFN-γ in cell culture supernatants was assayed by ELISA according to manufacturer's recommendations (Human IFN gamma ELISA Ready-SET-Go Kit, affymetrix). To estimate EC_{50} (the amount of peptide leading to 50% of maximum IFN-γ

secretion), IFN-γ secretion by each T cell clone was analyzed via nonlinear regression using Prism version 6.0 (GraphPad). In addition, IFN-γ release by KLL-specific clonotypes was measured after incubation with three MCPyV+, HLA-A*02+ MCC cell lines (WaGa, MS-1, and MKL-2). Cell lines were stimulated with IFN-β (Betaseron, BayerHealthCare; 3,000 U/mL) for 24 hours to induce expression of HLA class I, followed by 24 hours of culture after IFN-β washout. $2x10^4$ clonal KLL-specific T cells were incubated with $5x10^4$ cells from each MCC cell line, +/- IFN-β treatment, and incubated for 36 hours. Supernatants were assayed by ELISA as described above.

Cytokine Release with Large T-Ag transfected Targets**:** T cell clones were incubated with antigen presenting cells transiently transfected with plasmids encoding HLA-A*02:01 and GFP-truncated Large T-Ag (tLTAg) fusion protein (pDEST103-GFP-tLTAg). pDEST103-GFP-tLTAg was created using Gateway recombination cloning technology (Gateway) to insert tLTAg from pCMV-MCV156[37] into pDEST103-GFP. $3x10^4$ COS-7 cells were plated in flat-bottom 96-well plates in DMEM + 10% FBS, 200 nM L-glutamine and 100 U/ml Penicillin-Streptomycin. 24 hours later, wells were transfected using FuGENE HD (Promega) at a 6:1 ratio of transfection reagent to DNA with 25 ng HLA-A*02:01 and limiting dilution of pDEST103-GFP-tLTAg (25-0.08 ng) plus irrelevant DNA (pcDNA-6/myc-His C, Gateway) to a total of 25 ng. 48 hours after transfection, 10^4 viable KLL-specific T cells in TCM were added to target wells in duplicate. After 36 hours, supernatants were assayed by ELISA for IFNγ secretion and EC_{50} calculated as above. Transfected COS-7 cells were harvested at 48 and 72 hours post-transfection to quantitate transfection efficiency via percentage of cells both HLA-A*02 and GFP positive by flow cytometry.

RESULTS:

CD8+ T cells recognizing the MCPyV epitope KLLEIAPNC presented by HLA-A*0201 can be detected in a minority of HLA-matched MCC patients. HLA-A*02 is a prevalent HLA-type present in ~55% of our MCC cohort (n=97 low-resolution HLA class I typed patients; HLA-A*02:01 is the dominant A02 allele). We detected A*02-restricted T cell responses in MCC patients to an epitope of the common T-Ag (aa 15-23) in 14% of PBMC (10 of 69) and 21% of cultured TIL (5 of 24; TIL were expanded with mitogen/cytokine for 2 weeks[17]) from HLA-A*02+ patients. No tetramer+ cells were detected in PBMC from healthy HLA-matched controls (0 of 15, **Figure 1**). Among HLA-A*02+ patients, neither MCC-specific survival nor recurrence-free survival were significantly different between patients with or without detectable KLL-specific tetramer+ T cells (p= 0.593 and p=0.643, data not shown). We believe that the detected KLL-specific T cells were primed by MCPyV due to the limited homology between this epitope and the homologous region of other polyomaviruses known to infect humans (**Supplemental Table 1**). Moreover, this epitope is predicted to bind to HLA-A*02 ~100x better than these homologous peptides (IC_{50} for the KLL MCPyV peptide is 299 nM versus 6950-25799 nM for all other homologs as determined by the Immune Epitope Database[38]).

Characteristics of patients with KLL-specific T cells. Twelve patients had robust populations of KLL tetramer+ cells (>0.04% of CD8+ T cells) in their PBMC and/or cultured TIL. All patients were Caucasian, with a median age of 65 (range 50-77). The patients presented at varying stages of disease. Some developed progressive disease

and others showed no evidence of disease after definitive treatment during a median follow up period of 2.7 years (range 1.1 - 6.0) years. Patient demographics and relevant disease metrics are summarized in **Supplemental Table 2**.

Next generation sequencing of sorted KLL tetramer+ T cells reveals significant clonotypic diversity within and between patients. We sequenced the complementarity determining region 3 (CDR3) region of *TRB* of KLL tetramer-sorted cells from PBMC (n=9) and/or TIL (n=5) from 12 patients (**Figure 2)**. Paired KLL tetramer+ T cells from both PBMC and TIL were available for two patients. Out of 397 unique *TRB* sequences, only one public TCRβ clonotype was detected and shared between just two patients. This clonotype dominated the KLL-specific repertoire of these patients (59.1 or 21.5% of KLL-specific *TRB* sequencing reads). Complete TCRβ sequence results for each patient, in order of decreasing frequency, are in **Supplemental Table 3.** The diversity of the tetramer+ *TRB* repertoire varied greatly between patients. *TRB* diversity was not correlated with the frequency of tetramer+ T cells among total CD8+ cells in PBMC (**Figure S1**). We determined the clonality of each tetramer+ sample from PBMC (range: 0-1 with a completely clonal sample =1; see Methods for details) and found that there was no significant difference in MCC-specific survival or recurrence-free survival between patients with a less clonal (clonality <0.3, n=6) or more clonal (clonality >0.3, n=3) KLL-specific repertoire in their PBMC (**Figure S2**, p=0.52 and p= 0.81 by log-rank test).

T cell repertoire within matched tumor samples assessed via TCRβ sequencing

and immunohistochemistry. Archival tumor samples were available from 11 of 12 patients. When possible, primary tumors were acquired (n=6). For four patients with an unknown primary who presented with nodal disease, lymph nodes were analyzed. Two patients' primary tumors had insufficient material for study and we therefore analyzed a nodal tumor (present at time of diagnosis) from one patient and a metastasis (corresponding to time of PBMC collection) from the second patient. Tumors were assessed via immunohistochemistry (IHC) for viral status; HLA-I expression; and CD8+, CD4+ and FoxP3+ T cell infiltration (**Figure S3A**). All patients were robustly positive for MCPyV Large T-Ag protein by IHC. CD8+ cells were more predominant than CD4+ or FoxP3+ T cells in the majority of samples. *TRB* CDR3 of all T cells in each tumor sample were sequenced and total unique TCRβ clonotypes/tumor were plotted in **Figure S3A** (n=12, range=16-41,645 unique clonotypes/tumor).

We measured whether having a greater number of total T cells was associated with a survival benefit. *A priori*, patients were binned by whether their tumors had many infiltrating T cells (≥0.8 T cells/ng tumor DNA, n=7) or few T cells <0.3 T cells/ng tumor DNA, n=3); there was no survival difference between these two groups of patients **Figure S3B,** p=0.59 by log-rank test). In addition, we calculated the *TRB* clonality of each tumor analyzed. Increased clonality of the immune infiltrate within tumors is thought to represent an enrichment of cancer antigen-specific T cells and has been associated with improved response to immunotherapy[39]. There was no significant difference in MCC-specific survival or recurrence-free survival between patients with a less clonal repertoire in their tumors (clonality <0.1, n=7) versus those with a more clonal repertoire (clonality >0.1, n=4; **Figure S4A-B**, p=0.50 and p=0.64 by log-rank

test).

Increased frequency of intratumoral KLL-specific T cells is associated with MCC-specific survival. We next assessed how frequently KLL-specific T cells infiltrated MCC tumors. KLL-specific clonotypes within tumors were identified by determining the intersection between TCRβ CDR3 aa sequences in the tetramer-sorted sample (from **Figure 2**) and whole tumor samples from each patient. KLL-specific T cells constituted between 0-18% of the T cell repertoire of each tumor based on the total number of T cell genomes sequenced (n=12, mean 6.3%, s=5.8, **Figure S5A**). Tumors contained between 0-108 unique KLL-specific TCRβ clonotypes (mean = 19.4, s=32, **Figure S5B**). The rank (based on frequency) of each KLL-specific clonotype within each tumor was plotted; individual clonotypes ranged between being the most prevalent clonotype to very rare within each autologous tumor. KLL-specific clonotypes appeared to be more abundant (based on total percentage of all KLL-specific T cells in tumor) and predominant (based on percentage of individual KLL-specific clonotypes) in patients alive at time of last follow up or death (**Figure 3A**). Patients were binned *a priori* based on percentage of tumor with KLL-specific T cells. MCC-specific survival was significantly increased for patients who had a higher (1.9-18%; *n*=7) versus lower (0-0.14%; *n*=2) percentage of KLL-associated T cells in tumor (**Figure 3B,** p=0.0009 by log-rank test).

In addition, we asked whether the number of unique KLL-specific TCRβ CDR3 clonotypes infiltrating tumors was associated with survival. Indeed, there was a significant survival advantage among patients who had more (5-108, *n*=7) unique KLL-specific clonotypes in their tumors, compared to patients with few (0-3, *n*=4) KLL-

specific clonotypes (**Figure 3C,** p=0.0051). Lastly, we assessed whether these differences in frequency or diversity of KLL-specific T cell infiltration were associated with differences in recurrence-free survival. There was a trend for increased recurrence-free survival among patients with a higher versus lower frequency of KLL-specific T cells within tumors (**Figure 3D**; p=0.45), and among patients with more versus fewer KLL-specific clonotypes within tumors (**Figure 3E**; p=0.20).

When patients were separated into those who did and did not recur, the frequency of KLL-specific T cells was higher in tumors from patients without disease recurrence (median 10.4%) compared to patients who did recur (median of 3.2%, **Figure 4A,** p=0.11). In addition, the diversity of unique KLL-specific clonotypes was significantly higher in patients who did not recur (median of 38 clonotypes) compared to patients who did recur (median of 2 clonotypes; **Figure 4B,** p=0.02). Lastly, there is a trend toward increased survival after first metastasis in patients with more frequent (>1.9%) versus rare (< 0.14%) KLL-specific cells within their tumors, and this difference is significant compared to a historical cohort of 179 patients (**Figure S6,** p=0.01 by log-rank test).

Collectively, these data show that there is a significant survival advantage for patients for whom biopsies contain a higher relative percentage of KLL-specific T cells. Separately, a diverse intratumoral infiltration of KLL-specific T cells is beneficial.

TCRα/β sequence diversity among KLL-specific CD8+ T cell clones. To gain insight into functional differences of unique KLL-specific TCRs, we generated KLL-specific T cell clones from MCC patients' PBMC (n=4) and/or *ex vivo* tumor digest (n=1)

by sorting KLL-tetramer+ cells followed by limiting dilution cloning. Diversity of the TCRVβ of several KLL-reactive clones per patient was initially studied with fluorescent anti-TCRVβ antibodies via flow cytometry, and clones encompassing the spectrum of TCRVβ usage were expanded for further study. We determined the V, J and CDR3 sequences of both TCRα and β chains for 120 clones and identified 42 distinct clonotypes recognizing this epitope between 4 patients (**Table 1**). Among many private TCRα chains sequenced, one public TCRα chain using TRAV12-1*01 and encoding the CDR3 "CVLNNNDMRF" was found among clones from three of four patients.

KLL-specific clones display a hierarchy of functional avidity as measured by IFNγ secretion. To investigate functional differences among MCPyV-specific T cell clones, we measured secretion of a canonical Th1 effector cytokine, IFNγ**,** after stimulation with T2 target cells pulsed with limiting dilution of an alanine-substituted variant of the peptide (KLLEIAPNA; this peptide is still antigenic but less susceptible to oxidation, allowing direct comparisons of T cell clones to each other. See methods for details). Clones displayed narrow ranges of intra-patient variability for functional avidity (**Table 1 and Figure 5A**). Concordant results were obtained in a separate but analogous assay using targets transfected with limiting dilution of a plasmid encoding truncated Large T-Ag (**Figure 5B**). Importantly, patients with improved MCC-specific survival had more functionally avid T cell clonotypes ($p < 0.05$). To further interrogate the effector function of these clonotypes, we tested the ability of 28 unique KLL-specific clonotypes to recognize the MCPyV+, HLA-A*02+ MCC cell lines (WaGa, MS-1 and MKL-2) +/- IFNβ treatment. Five unique clonotypes secreted IFNγ when incubated with MS-1; no clones

recognized WaGa or MKL-2 (**Table 1 and Figure 5C).** Reactive clones were derived from patient w678 who had the most functionally avid clonotypes based on the IFN-γ release assay. Lastly, we compared the ability of KLL-specific clonotypes to bind to both wild type and 'CD8 independent' tetramers containing mutations in HLA-A*02:01 to abrogate CD8 stabilization of the TCR:pMHC interaction, which may select for more avid TCRs[31,32]. While clonotypes that were more functionally avid in the IFNγ assay often bound both wild type (WT) and CD8 independent tetramers similarly, other avid clonotypes did not bind the CD8 independent tetramer well **(Table 1 and Figure 5D)**, suggesting this assay could be used to further narrow down candidate TCRs for transgenic TCR (tTCR) therapy and/or select TCRs also suitable for generating CD4+ tTCRs. No significant correlations between clonotype avidity and enrichment within tumors were identified.

DISCUSSION:

The purpose of our study was to characterize the TCR repertoire restricted to a naturally processed epitope of MCPyV in the context of the prevalent HLA-A*02 allele, and to assess whether differences in either the breadth or avidity of the TCRs correlated with the effectiveness of the T cell response *in vivo*. We identified KLL tetramer+ CD8+ T cells in a minority of HLA-matched MCC patients, and did not find survival or recurrence differences between HLA-A*02+ patients with or without detectable circulating tetramer+ T cells. The TCRβ repertoires of the KLL-specific T cells were strikingly diverse. A higher frequency of KLL-specific T cells within tumors, as identified by their signature *TRB* CDR3 sequences, was associated with a significant MCC-specific survival advantage. These findings identify the diversity of the CD8+ T cell response to MCC, and create a target for therapeutic maneuvers to boost tumor immunity. Overall, our outcome correlation findings provide a rationale for active or passive immunization to increase MCPyV-specific CD8+ T cell diversity and avidity, and for manipulations of the immunosuppressive tumor microenvironment to promote the infiltration by these T cells.

This study is, to our knowledge, the first to study the TCR repertoire restricted to any epitope of MCPyV. It is also one of the first studies to utilize this high-throughput TCR sequencing approach, which has revealed diversity that may have been missed in historically traditional methods. TCR sequencing was accompanied by the generation of KLL-specific clones and functional avidity characterization in a physiologically relevant system, allowing for paired analysis of unique TCRs and matched T cell clones. Similar

approaches have been utilized in other virally associated cancers to select the 'best' TCRs for transgenic T cell therapy[40], a therapeutic modality that has merit in MCC.

The frequency of KLL-specific T cells among A*02+ MCC patients has never been studied and is lower than might be expected (only 14% of A*02+ MCC patients have detectable KLL-specific T cells), compared to our finding that T cells restricted to an HLA-A*2402-epitope are found in 64% of HLA-matched PBMC[18]. These findings could possibly be the result of poor processing and presentation of the KLL epitope (for instance, the A*2402 restricted epitope has a 2x lower IC_{50} for cognate HLA compared to the KLL epitope by IEDB[38]), which would prevent patients from being able to mount an immune response. Indeed, this hypothesis is supported by our finding that functionally avid KLL-specific clonotypes were only able to recognize one of three tested MCPyV+ A*02+ cell lines, even after upregulation of MHC-I on all three cell lines.

Alternatively, there may be adequate antigen presentation and yet patients are unable to mount their own endogenous effector response to this epitope (due to local immunosuppression or a host of other factors). These patients in particular may benefit from therapy with T cells expressing transgenic TCRs (tTCRs) restricted to this epitope. Importantly, TCR sequences generated from this study may be used to create useful reagents (ie, tTCR clones) to detect and quantify KLLEIAPNC on the surface of primary MCCs to help distinguish between these hypotheses.

In this first in-depth examination of MCPyV-specific TCRs, we found that out of 397 KLL-specific TCRs detected among 12 patients, the vast majority (396) were private (only observed in one patient), with only one public TCR observed in two individuals. Public TCRs have been observed in multiple species in response to many viral

infections and tumor antigens[41], and might be expected to be particularly prevalent in the response to a DNA virus thought to have low mutational capacity such as MCPyV. However, that idea is discordant with our observation in this study of a predominantly private repertoire, although the small sample size of our study (*n*=12) is a limitation. Increased diversity of an antigen-specific T cell response led to improved outcomes in models of chronic infections such as CMV[42] and herpes[43]. In our study, no association between the diversity of the circulating KLL-specific T cell repertoire and MCC outcomes was detected; however, an increased number of KLL-specific clonotypes within tumors was associated with improved MCC-specific survival. While these studies elucidate TCR diversity restricted to a single epitope of MCPyV, we now have validated tetramers for 5 other peptide/HLA combinations (unpublished observations) and could replicate these studies to assess whether this striking diversity in immune response is specific to the KLL epitope or more broadly observed in the CD8+ T cell response to MCPyV.

Our finding that a higher frequency of KLL-specific T cells within primary tumors is associated with a significant MCC-specific survival advantage builds on previously published work that CD8+ infiltration into tumors is associated with improved survival[14,15], but is the first to confirm the importance of MCPyV-specific T cells in the infiltrate. In contrast, the presence of detectable circulating KLL-specific T cells was not associated with improved MCC-specific survival. Therefore, efforts should be focused toward improving the tumor homing and infiltration of both endogenous and therapeutic MCPyV-specific T cells. Candidate agents include those that reverse silencing of Th1-chemokines by epigenetically modulating tumor cells, which have been shown to

increase T cell infiltration as well as the effectiveness of both checkpoint inhibitors blockade and adoptive T cell therapy in mouse models of ovarian cancer[44]. In addition, downregulated E-selectin on intratumoral blood vessels has been correlated with poor CD8+ infiltration in MCCs[45], and agents that block generation of reactive nitrogen species- one mechanism of E-selectin downregulation- may help reverse this immune escape.

In parallel to other infections and malignancies[22-26], we hypothesized that increased avidity of the TCR repertoire may be correlated with the effectiveness of the T cell response *in vivo*. Forty-two distinct clonotypes recognizing this epitope were identified by creating clones from PBMC or tumor of four patients, and clones generally expressed a narrow intra-patient range of functional avidities with more avid clones isolated from patients with better MCC-specific outcomes. Several clonotypes appeared more avid than others in four unique assays and may be suitable TCR candidates for transgenic therapeutic T cells.

There are several limitations to our study. Our sample size was limited by the number of subjects with this rare cancer in our research cohort who had tetramer+ T cells (*n*=12). Because there are no durable treatments for advanced disease, patients went on to receive a variety of therapies including chemotherapy, radiation, and immunotherapies that were not standardized between patients. Notably, all patients in this study who received no further therapy since definitive excision of their presenting lesion (n=4) are currently alive with no evidence of disease (median follow up time of 2.9 years; average recurrence for most MCC patients is 9 months), supporting the importance of their immune systems in fighting MCC. Another limitation is that we

studied only a single MCPyV epitope. It is almost certain that MCPyV-specific T cells recognizing other epitopes besides KLL contributed to the anti-tumor immune response of each patient. However, KLL specific T cells were among the top 10 most frequent clonotypes within tumors of 7 of 9 patients studied, strongly suggesting that these T cells are a predominant factor in the effector response to MCC. Lastly, we extrapolated the frequency of KLL-specific T cells within tumors based on the frequency of KLL-specific sequencing reads, not the frequency of T cells, which may have introduced a degree of unbiased error in our analysis.

In summary, MCPyV-specific CD8+ T cells are detectable *ex vivo* in a substantial portion of HLA A*02(+) MCC patients and have considerable TCR diversity with a hierarchy of clonal avidity. Our hypothesis that infiltration of MCPyV-specific T cells leads to superior tumor control is supported by our findings of increased MCC-specific survival in patients with a higher frequency of KLL-tetramer + T cells. Our findings support further investigation of agents that improve T cell tumor homing and infiltration, as well as use of avid TCRs for transgenic T cell therapy in advanced MCC.

FIGURE LEGENDS:

Figure 1: KLL tetramer+ CD8+ T cells detected in 14% of PBMC and 21% of TIL from MCC patients. MCPyV-specific T-cell frequencies among HLA-A*02+ patients (n=69 for PBMC, 24 for TIL) or PBMC from control subjects (n=15). PBMC acquired when patients had evidence of disease was used in all analyses. Mean for each group is depicted, with dashed line at threshold for credible responses. The mean frequency of tetramer+ CD8+ cells was significantly different between MCC patient PBMC and control subjects (p=0.0004 by Mann Whitney test) but not significantly different between MCC patient TIL and control PBMC (p=0.11).

Figure 2: *TRB* CDR3 clonotype diversity among KLL tetramer+ CD8+ cells from PBMC and TIL of 12 patients. KLL tetramer + CD8+ T cells were sorted by flow cytometry (a representative plot is shown) and the CDR3 region from *TRB* was sequenced. All productive *TRB* clonotypes with an estimated number of genomes ≥ 2 within each sample are indicated in proportion to their prevalence with a pie chart, with total T cells sequenced at bottom right in each pie. Patients are identified by unique "w" or "z" number. Among 397 total *TRB* clonotypes, only one shared clonotype was detected among two patients (highlighted in yellow). Paired tumor and PBMC samples were available for two patients (boxed).

Figure 3: Increased tumor infiltration of KLL-specific clonotypes is associated with improved MCC-specific survival. (**A**) A wedge representing the total number of productive unique clonotypes/tumor are plotted for each tumor on a log scale. Each tumor is identified by patient "w" or "z" number and type of tumor. KLL-specific

clonotypes (yellow) are depicted within each tumor with a width approximately proportional to their frequency within each tumor. More predominant clonotypes are located to the left for each tumor. The number of KLL-specific clonotypes out of the total number of unique clonotypes is tabulated at far right. Wedges for tumors from patients alive at time of sensor are in green, and wedges for tumors in grey are from patients who have died of MCC. (**B**) MCC-specific survival was significantly increased for patients who had higher (n=9) versus lower (n=2) percentage of KLL-specific T cells in tumor (1.9-18 % versus 0-0.14%, p=0.0009 by log-rank test). (**C**) MCC-specific survival was increased for patients who had many (5-108, n=7) unique KLL-specific clonotypes in their tumors, compared to patients with few KLL-specific clonotypes (0-3, n=4, p=0.0051 by by log-rank test) (**D**) There was no significant difference in recurrence-free survival between patients with a higher versus lower frequency of KLL-specific T cells (patients binned as in Figure 3B; p=0.4492 by log-rank analysis). (**E**) Patients who had many KLL-specific clonotypes (5-108, n=7) had a trend toward better recurrence-free survival compared to patients with intermediate or few tetramer+ clonotypes (0-3, n=4, p=0.1369 by log-rank test). LN = lymph node; UP= unknown primary; 1° = primary lesion; Met = metastasis.

Figure 4: Patients without disease recurrence have a greater frequency and number of KLL-specific clonotypes in their tumors. Patients were grouped by whether they developed metastatic disease (n=7) or remained disease-free after definitive treatment of first presentation of disease (n=3). (**A**) The percentage of KLL-specific T cells was higher in patients without recurrence (range 4.3-18%) compared to

those who developed metastatic disease (range 0-10.8%, p=0.11). (**B**) The number of KLL-specific clonotypes was significantly higher in patients without recurrence (median 38, range 9-108) compared to those who developed metastatic disease (median 2, range 0-17, p= 0.02).

Figure 5: Functional avidity of 28 KLL-specific clonotypes from 4 patients. EC_{50} values for IFN-γ secretion by KLL-specific clones in response to limiting dilution of peptide (**A**) or limiting dilution of tLT-Ag DNA (**B**) are plotted for each patient, with mean of all clones/patient depicted. For replicate experiments of clones with the same TCR, the mean EC_{50} is plotted. Clonotypes from the same patient generally had similar functional avidities; more avid clonotypes are detected among patients with better MCC-specific survival. Statistical comparisons were made between patients; *, $p < 0.05$; **, $p < 0.01$, Mann Whitney test. **(C)** Clonotypes from one patient respond to the MCPyV+, HLA-A02+ MCC cell line MS-1 +/- IFN-β treatment to upregulate HLA-I. Mean of duplicates + SEM are shown after subtracting background IFN-γ secretion by T cells without targets; representative results from one of at least two separate experiments with each clone are shown. **(D)** Select clonotypes are able to bind a 'CD8 independent' KLL-tetramer.

Figure 1

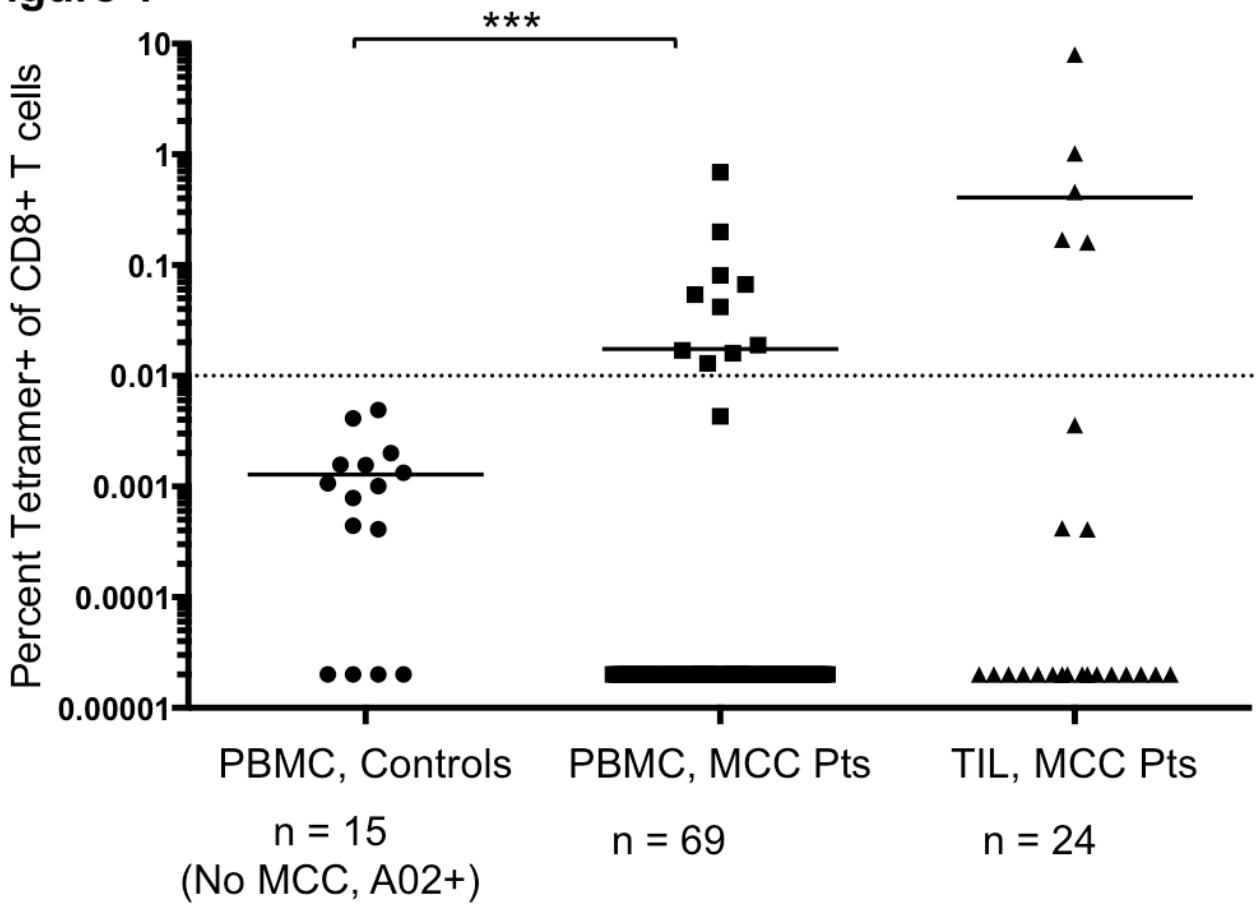
***
Percent Tetramer+ of CD8+ T cells
10
1
0.1
0.01
0.001
0.0001
0.00001
PBMC, Controls
n = 15
(No MCC, A02+)
PBMC, MCC Pts
n = 69
TIL, MCC Pts
n = 24

Figure 2

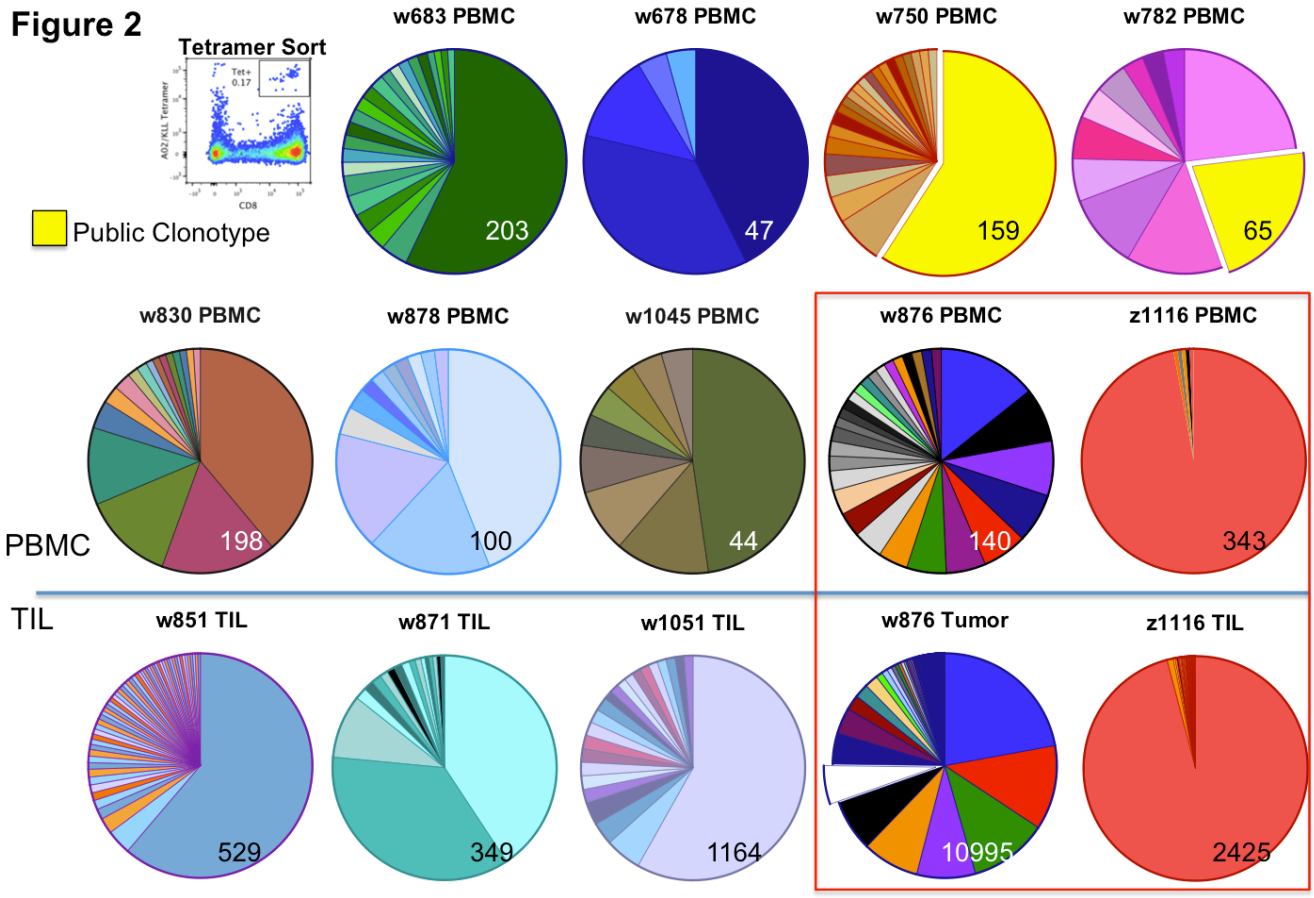
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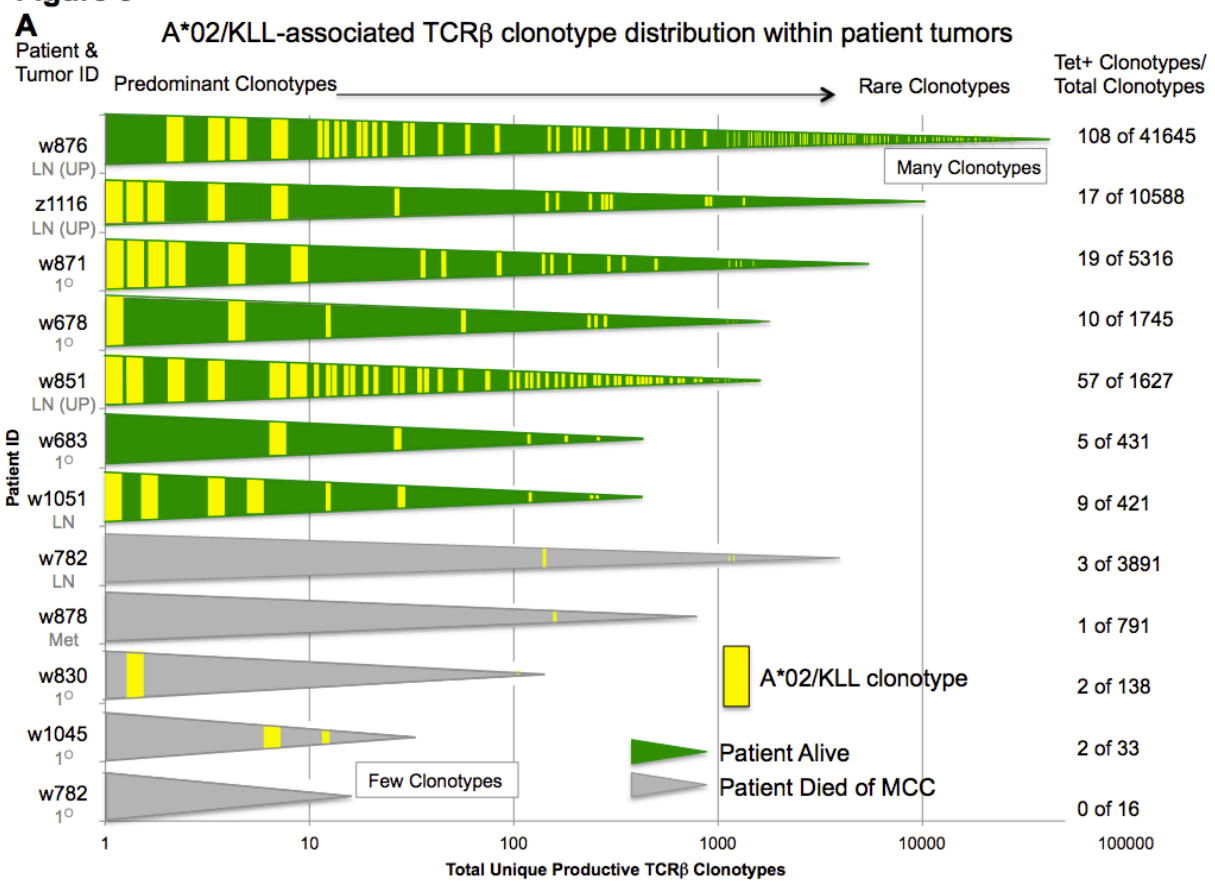
Figure 3


A
A*02/KLL-associated TCRβ clonotype distribution within patient tumors
Patient & Tumor ID
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Rare Clonotypes
Tet+ Clonotypes/ Total Clonotypes
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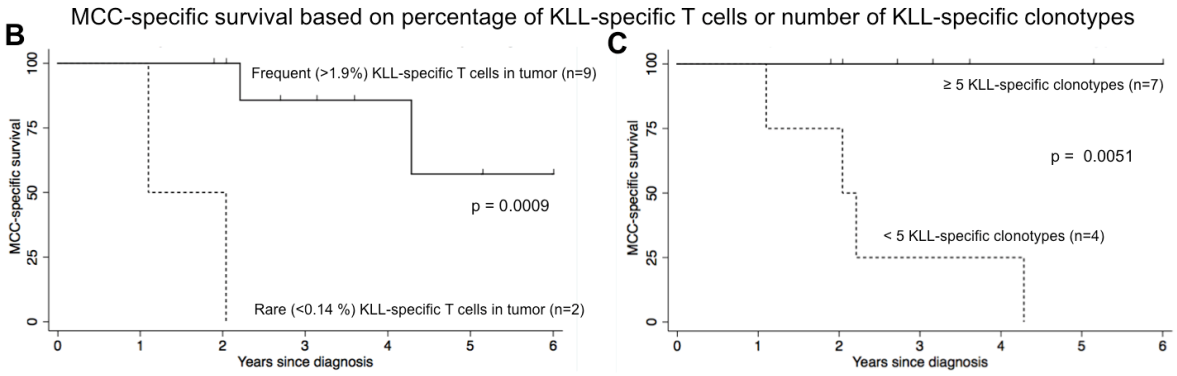
z1116 LN (UP)
w871 1°
w678 1°
w851 LN (UP)
w683 1°
w1051 LN
w782 LN
w878 Met
w830 1°
w1045 1°
w782 1°
Patient ID
Many Clonotypes
Few Clonotypes
A*02/KLL clonotype
Patient Alive
Patient Died of MCC
108 of 41645
17 of 10588
19 of 5316
10 of 1745
57 of 1627
5 of 431
9 of 421
3 of 3891
1 of 791
2 of 138
2 of 33
0 of 16
1
10
100
1000
10000
100000
Total Unique Productive TCRβ Clonotypes


MCC-specific survival based on percentage of KLL-specific T cells or number of KLL-specific clonotypes
B
Frequent (>1.9%) KLL-specific T cells in tumor (n=9)
p = 0.0009
Rare (<0.14 %) KLL-specific T cells in tumor (n=2)
MCC-specific survival
Years since diagnosis
C
≥ 5 KLL-specific clonotypes (n=7)
p = 0.0051
< 5 KLL-specific clonotypes (n=4)
MCC-specific survival
Years since diagnosis

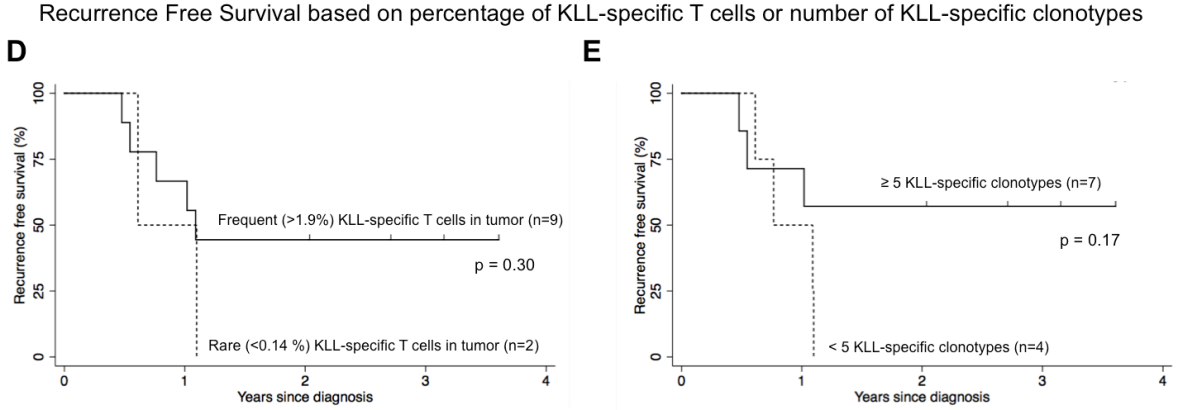

Recurrence Free Survival based on percentage of KLL-specific T cells or number of KLL-specific clonotypes
D
Frequent (>1.9%) KLL-specific T cells in tumor (n=9)
p = 0.30
Rare (<0.14 %) KLL-specific T cells in tumor (n=2)
Recurrence free survival (%)
Years since diagnosis
E
≥ 5 KLL-specific clonotypes (n=7)
p = 0.17
< 5 KLL-specific clonotypes (n=4)
Recurrence free survival (%)
Years since diagnosis

Figure 4

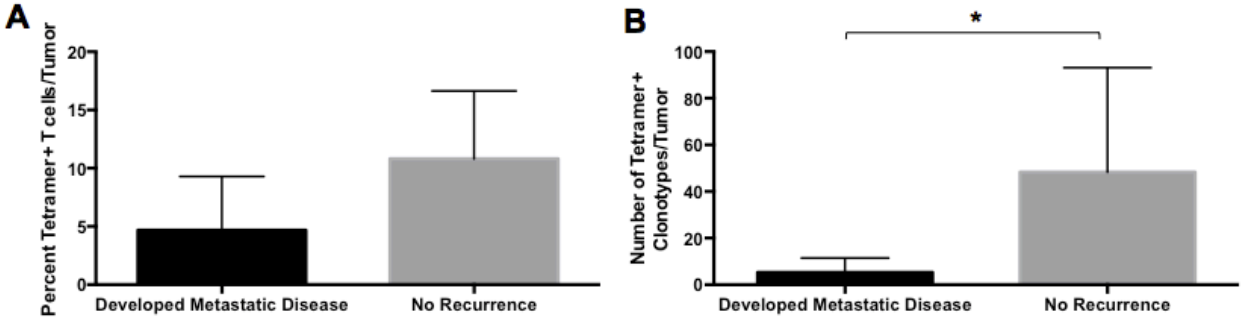

A
Percent Tetramer+ T cells/Tumor
20
15
10
5
0
Developed Metastatic Disease
No Recurrence
B
*
Number of Tetramer+ Clonotypes/Tumor
100
80
60
40
20
0
Developed Metastatic Disease
No Recurrence

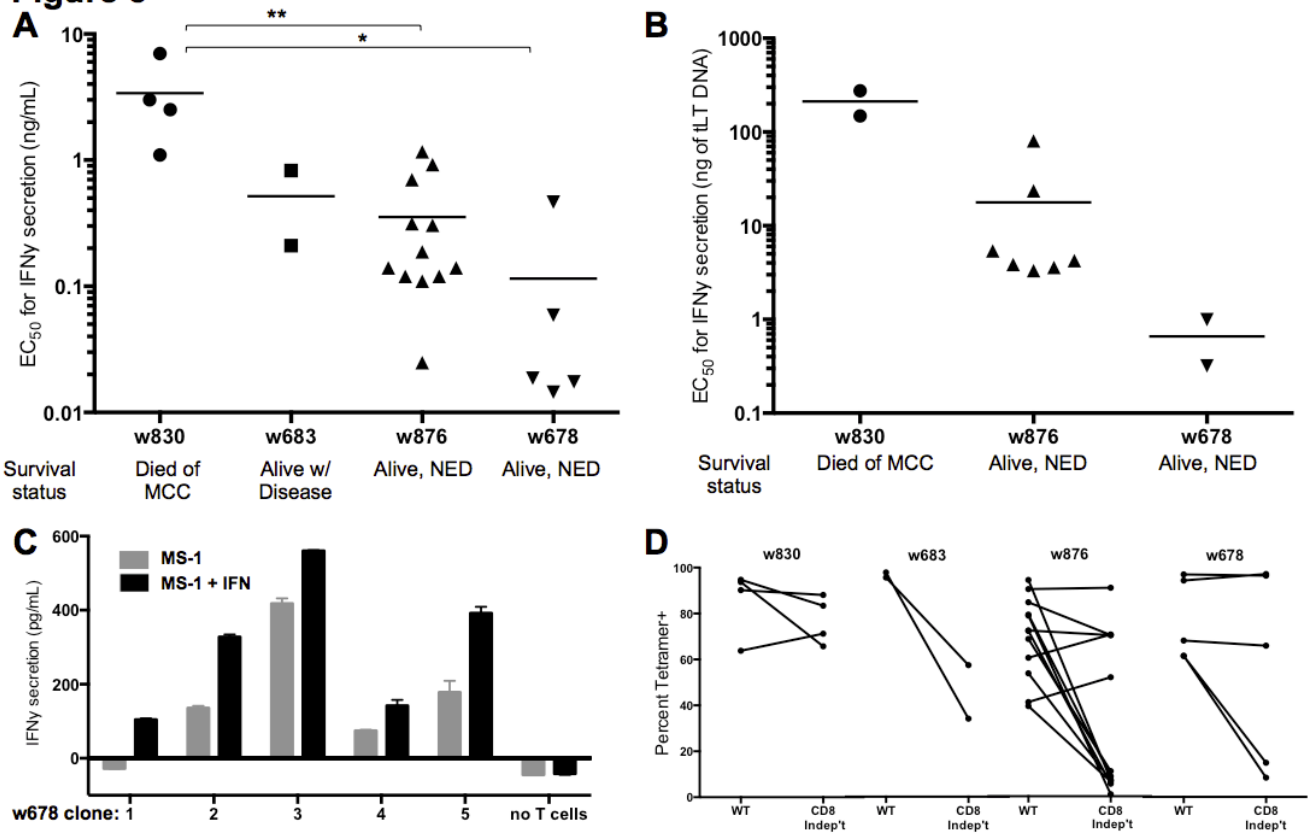
Table 1: TCRα/β sequences of HLA-KLL tetramer + clones from four MCC patients

	Alpha Chain			Beta Chain			Functional Assays			
Pt	V gene	CDR3 region	J gene	V gene	CDR3 region	J gene	EC_{50} (ng/mL peptide)	EC_{50} (ng/uL DNA)	Recog. MS-1?	Mutant Tet+?
w830 Clonotypes										
1	TRAV24*01	CAFNTDKLIF	TRAJ34*01	TRBV12-4*01	CASSLAGFRFF	TRBJ2-1*01	4.6 0.4		No	Lower
2	TRAV38-1*01	CALTSGSRLTF	TRAJ27*01	TRBV19*01	CASSIMLYSNQPQHF	TRBJ1-5*01	12 1.6	140	No	Equal
3	TRAV38-1*01	CAYPSTDKLIF	TRAJ34*01	TRBV19*01	CASSILGASNQPQHF	TRBJ1-5*01		270		Equal
4	TRAV12-1*01	CVLNNNDMRF	TRAJ43*01	TRBV19*01	CASSILGASNQPQHF	TRBJ1-5*01	1.1		No	Equal
5	TRAV12-1*01	CVVNANDMRF	TRAJ43*01	TRBV10-3*01	CAIRARDQNTGELFF	TRBJ2-2*01	5.1 0.89		No	Equal
w683 Clonotypes										
1	TRAV12-1*01	CVVALYSGGGADGLTF	TRAJ45*01	TRBV6-5*01	CASRSQNYYGYTF	TRBJ1-2*01	1.9 0.36 0.26		No	Lower
2	TRAV12-1*01	CVLNNNDMRF	TRAJ43*01	TRBV6-5*01	CASRSQNYYGYTF	TRBJ1-2*01	0.21		No	Lower
w678 Clonotypes										
1	TRAV12-1*01	CVLNNNDMRF	TRAJ43*01	TRBV10-3*01	CAIRQFDANTGELFF	TRBJ2-2*01	0.43 0.50 0.47	0.32	Yes	Equal
1.5		UNKNOWN*		TRBV10-3*01	CAIRQFDANTGELFF	TRBJ2-2*01	0.84		Yes	Equal
2	TRAV38-1*01	CAFRVSHDMRF	TRAJ43*01	TRBV19*01	CASSIIAGSSYNEQFF	TRBJ2-1*01	0.017 0.012		Yes	Lower
3	TRAV12-1*01	CVVATYSGGGADGLTF	TRAJ45*01	TRBV19*01	CASSIIAGSSYNEQFF	TRBJ2-1*01	0.022 0.013	1.0	Yes	Equal
4	TRAV12-1*01	CVVATYSGGGADGLTF	TRAJ45*01	TRBV10-2*01	CASSSGNPSTDTQYF	TRBJ2-3*01	0.0094 0.028		Yes	Equal
5	TRAV38-1*01	CAFRVSHDMRF	TRAJ43*01	TRBV10-2*01	CASSSGNPSTDTQYF	TRBJ2-3*01	0.11 0.054		Yes	Lower
		UNKNOWN*			UNKNOWN*		0.060 0.058		Yes	Equal
w876 Clonotypes										
1	TRAV12-1*01	CVVGEYSGGGADGLTF	TRAJ45*01	TRBV28*01	CAIRAGASYNEQFF	TRBJ2-1*01	0.31	5.6		Lower
2	TRAV12-1*01	CVVTEYSGGGADGLTF	TRAJ45*01	TRBV10-3*01	CASRGQNTGELFF	TRBJ2-2*01	1.2		No	Lower
3	TRAV19*01	CALGGGTFTSGTYKYIF	TRAJ40*01	TRBV9*02	CASSVEDYTGELFF	TRBJ2-2*01	0.12	11	No	Equal
4	TRAV12-1*01	CVVYTGYSGGGADGLTF	TRAJ45*01	TRBV10-2*01	CASSVLNTGELFF	TRBJ2-2*01	0.31	14	No	Equal
5	TRAV38-1*01	CAYNQGGKLIF	TRAJ23*01	TRBV10-2*01	CASSVLNTGELFF	TRBJ2-2*01	0.11		No	Equal
6	TRAV12-1*01	CVVPLYSSASKIIF	TRAJ3*01	TRBV6-1*01	CASSDTPDLNTEAFF	TRBJ1-1*01	0.015 0.035		No	Lower
7	TRAV12-1*01	CVLNNNDRF	TRAJ43*01	TRBV6-1*01	CASSDTPDLNTEAFF	TRBJ1-1*01	0.14	3.6	No	Lower
8	TRAV12-1*01	CVVYASKIIF	TRAJ3*01	TRBV6-1*01	CASSDTPDLNTEAFF	TRBJ1-1*01		4.2		Lower
9	TRAV12-1*01	CVGNNNDMRF	TRAJ43*01	TRBV10-3*01	CAISARDQNTGELFF	TRBJ2-2*01	0.12 0.2449		No	Lower
10	TRAV12-1*01	CVVYGSSNTGKLIF	TRAJ37*02	TRBV10-3*01	CAIRRQDQNTGELFF	TRBJ2-2*01	0.7		No	Lower
11	TRAV12-1*01	CVVYTGYSGGGADGLTF	TRAJ45*01	TRBV10-3*01	CAIHEGDSNTGELFF	TRBJ2-2*01				Equal

12	TRAV3*01	CAVRDNSGTYKYIF	TRAJ40*01	TRBV7-2*04	CASSLAGLAGTDTQYF	TRBJ2-3*01				Lower
13	TRAV12-1*01	CVVTDTSGGGADGLTF	TRAJ45*01	TRBV7-2*04	CASSLAGLAGTDTQYF	TRBJ2-3*01		3.859		
14	TRAV12-1*01	CVVPSAGKSTF	TRAJ27*01	TRBV2*03	CASSEFAGQETQYF	TRBJ2-5*01		5.399		Lower
15		UNKNOWN*		TRBV6-5*01	CASRASNTYGYTF	TRBJ1-2*01		80.34	No	
16	TRAV38-1*01	CAYNQGGKLIF	TRAJ23*01	TRBV19*01	CASSTLSGTHNEQFF	TRBJ2-1*01	0.12		No	Lower
17	TRAV12-1*01	CVVYGSSNTGKLIF	TRAJ37*02	TRBV7-2*04	CASSLAGLANNEQFF	TRBJ2-1*01				Lower
18	TRAV14	CAMREAQSGGYQKVTF	TRAJ13*01	TRBV12-4*01	CASSFGSGTKDTQYF	TRBJ2-3*01				Equal
19	TRAV12-1*01	CVVYTGYSGGGADGLTF	TRAJ45*01	TRBV10-2*01	CASSGQNTGELFF	TRBJ2-2*01	0.92		No	Lower
20	TRAV10*01	CVVSAGINGADGLTF	TRAJ45*01	TRBV12-4*01	CASSPWDEQFF	TRBJ2-1*01				Lower
21	TRAV3*01	CAVRDNSGTYKYIF	TRAJ40*01	TRBV13*02	CASSSGTSGGLTYNEQFF	TRBJ2-1*01				
22		UNKNOWN*		TRBV13*02	CASSSRTKAYEQYF	TRBJ2-7*01				
23	TRAV12-3*01	CAMSVAQGGSEKLVF	TRAJ57*01	TRBV6-6*01	CASSYQIGLSYEQYF	TRBJ2-7*01				
24		UNKNOWN*		TRBV28*01	CASSFDSKGSNTGELFF	TRBJ2-2*01				
25	TRAV27*01	CAGDQGGSEKLVF	TRAJ57*01	TRBV16*01	CASSQLRTGDEYEQYF	TRBJ2-7*01				
26	TRAV12-1*01	CVVYTGYSGGGADGLTF	TRAJ45*01	TRBV13*02	CASSSGTSGGLNYNEQFF	TRBJ2-1*01				
27	TRAV3*01	CALTGYSTLTF	TRAJ11*01	TRBV19*01	CASRSQLAVLNEQFF	TRBJ2-1*01				
28		UNKNOWN*		TRBV28*01	CASRGGSSYNEQFF	TRBJ2-1*01				
29	TRAV12-1*01	CVVPLYSSASKIIF	TRAJ3*01	TRBV10-2*01	CASSVLNTGELFF	TRBJ2-2*01	0.12 0.16	3.326	No	Equal
30		UNKNOWN*		TRBV10-3*01	CATRDINTGELFF	TRBJ2-2*01				
		UNKNOWN*		TRBV6-1*01	CASSDTPDLNTEAFF	TRBJ1-1*01		24		

*select *TRA* or *TRB* sequences were unresolved with next-generation sequencing.

Figure 5


A
B
C
D
**
*
EC50 for IFNγ secretion (ng/mL)
EC50 for IFNγ secretion (ng of tLT DNA)
w830
w683
w876
w678
Survival status
Died of MCC
Alive w/ Disease
Alive, NED
MS-1
MS-1 + IFN
IFNγ secretion (pg/mL)
w678 clone: 1
2
3
4
5
no T cells
Percent Tetramer+
WT
CD8 Indep't

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Supplemental Data associated with "Merkel polyomavirus epitope-specific T cells express strikingly diverse T cell receptors and are correlated with improved survival upon infiltration into Merkel cell carcinomas."

SUPPLEMENTAL METHODS

Immunohistochemistry: FFPE embedded tumor tissue was stained (Experimental Histopathology at FHCRC) and slides scored by a dermatopathologist who was blinded to patient characteristics. Samples were stained with anti-CD8 (Dako, clone 144B at 1:100) and intratumoral CD8+ T cells (completely surrounded by tumor without neighboring stroma) on a scale from 0 (absent CD8+ cells) to 5 (>732 intratumoral CD8+ cells/mm^2) as described by Paulson *et. al*[14]. In addition, tumors were stained with anti-MHC class I[27] (MBL, clone EMR8-5) and CM2B4 to measure MCPyV T-antigen expression[28] (Santa Cruz, 1:50). Tumors were stained with anti-CD4 (Cell Marque clone SP35, 1:25) and anti-FoxP3 (eBiosciences clone FJK-16s, 1:25) and reported as the number of positive cells/mm^2.

T cell receptor clonality: A) Tetramer-sorted cells: Shannon entropy was calculated on the estimated number of genomes (≥2) of all productive *TRB* and normalized by dividing by the log2 of unique productive sequences in each sample. Clonality was calculated as 1- normalized entropy. B) Tumors: Clonality was calculated in the same method, using all *TRB* sequences in the sample to calculate normalized entropy.

SUPPLEMENTAL FIGURES

Table S1: Homologs to the CT15-23 (KLLEIAPNC) epitope from other polyomaviruses

T-Ag aa #	15	16	17	18	19	20	21	22	23		IC_{50} binding to HLA A*02 (nM)
VIRUS											
MCPyV	**K**	**L**	**L**	**E**	**I**	**A**	**P**	**N**	**C**		**299**
BKV	D	L	L	G	L	E	R	A	A		19316
JCV	D	L	L	G	L	D	R	S	A		19439
KIV	Q	L	L	C	L	D	M	S	C		6950
WUV	Q	L	L	G	L	D	M	T	C		7444
SV40	D	L	L	G	L	E	R	S	A		19586
HPyV6	D	L	I	G	L	S	M	A	C		19258
HPyV7	E	L	I	G	L	N	M	A	C		15594
TSV	D	L	L	Q	I	P	R	H	C		25799

Residues in grey boxes are highly divergent. While putative HLA 'anchor residues' 2 and 9 are conserved and may permit presentation of homologs by HLA-A*02, differences in TCR contact residues (middle of peptide) may be sufficient to reduce binding of homologs by MCPyV CT15-23 specific T cells. Homologs are much less likely to bind to human HLA-A*0201, based on IC_{50} values calculated via ANN using the online Immune Epitope Database Analysis Resource binding prediction tool.

Table S2: Characteristics of MCC Patients with A*02/KLL Tetramer+ T cells

Pt ID	Stage at Dx	Gender	Primary Site	Survival Status	Recurrence	Age at Dx	Tetramer+ Samples	Tetramer+ % of CD8s
w678	IIA	male	lower limb	alive	Local & Distant	64	PBMC	0.08
w683	IIA	male	lower limb	alive	LN & Distant	66	PBMC	0.69
w750	IIA	female	buttock	deceased	LN & Distant	58	PBMC	0.19
w782	IIIA	male	upper limb	deceased	Local & Distant	74	PBMC	0.05
w830	IIIA	male	head & neck	deceased	Local & Distant	58	PBMC	0.20
w851	IIIB	female	unknown	alive (NED)	No	77	TIL	0.16
w871	IA	male	buttock	alive (NED)	No	53	TIL	0.17
w876	IIIB	male	unknown	alive (NED)	No	50	PBMC	0.08
							TIL	7.98
w878	IV	female	unknown	deceased	N/A	54	PBMC	0.06
w1045	IIIA	female	head & neck	deceased	Distant	70	PBMC	0.02
w1051	IIIB	male	unknown	alive (NED)	No	70	TIL	0.43
z1116	IIIB	male	unknown	alive	Distant	67	PBMC	0.2
							TIL	1.04

Abbreviations: MCC, Merkel cell carcinoma; Pt, patient; Dx, diagnosis; NED, no evidence of disease; LN, lymph node; TIL, tumor infiltrating lymphocytes.

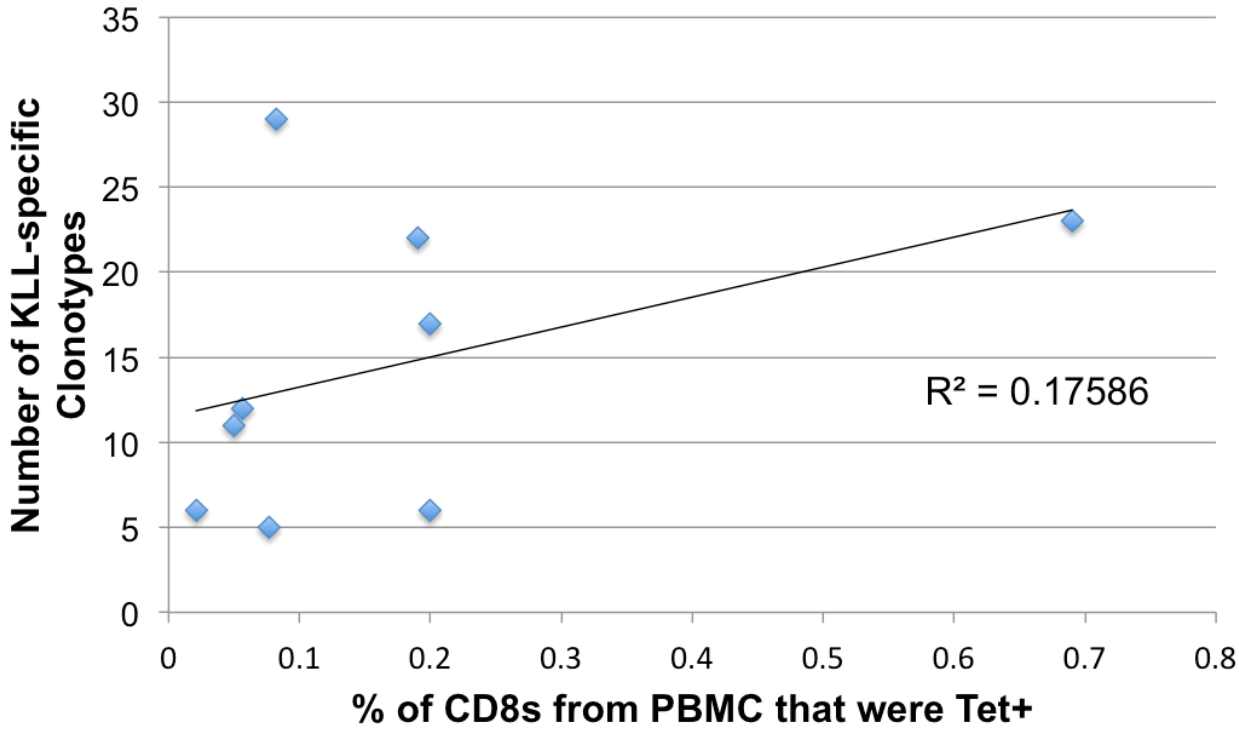


Figure S1: KLL-specific TCR diversity in PBMC is not correlated with the magnitude of KLL-specific responses. Number of unique clonotypes (present at ≥2 estimated number of genomes in each sample) was plotted against % of CD8+ cells positive for KLL-tetramer staining. No significant correlation was found ($r^2 = 0.17$).

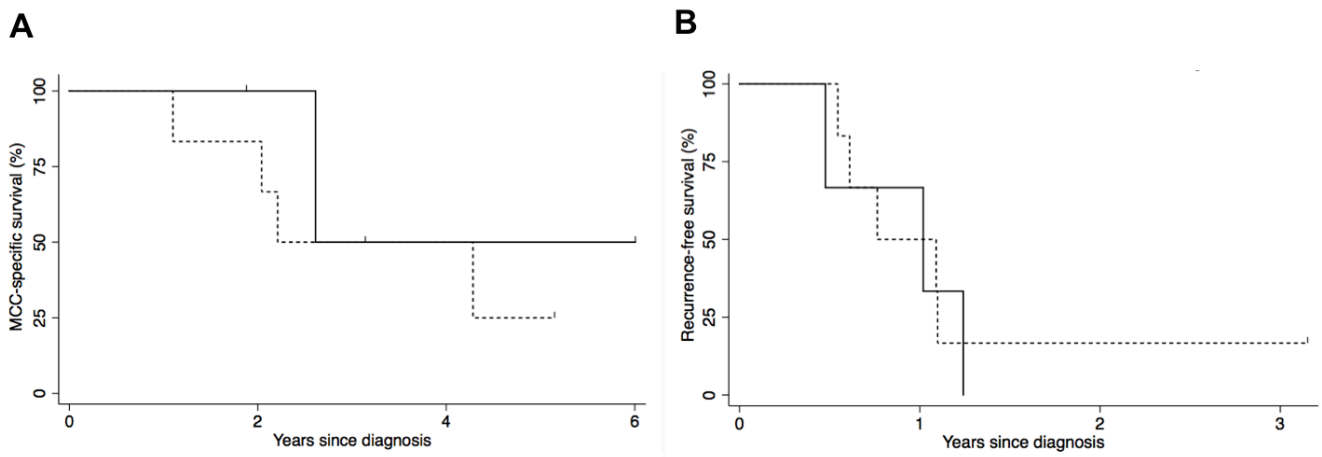
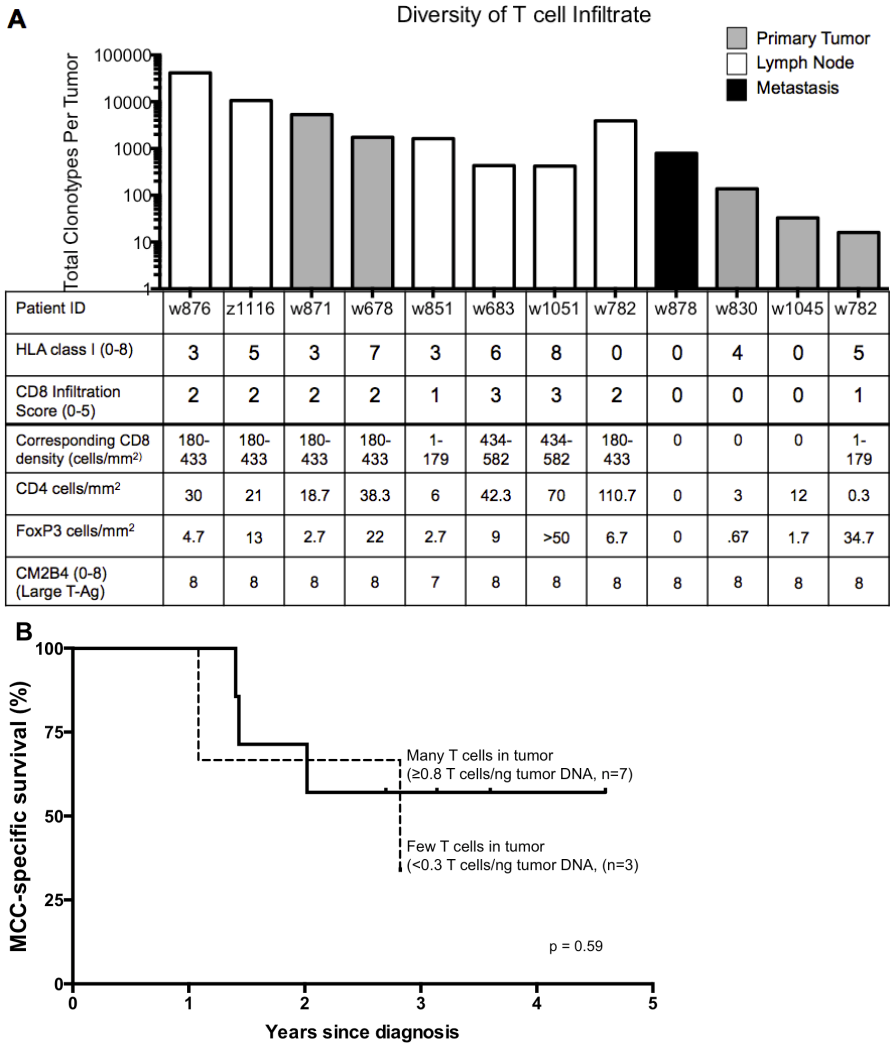


Figure S2: Clonality of KLL-specific T cell repertoire in PBMC of MCC patients does not correlate with disease outcome. Clonality of the KLL-specific repertoire from PBMC was calculated and patients were binned by high (>0.3, n=3) or low (<0.3, n=6) clonality. MCC-specific survival **(A)** or recurrence-free survival **(B)** between the two groups of patients were not significantly different by univariate analysis (p=0.52 or p=0.81 by log-rank test).



Patient ID	w876	z1116	w871	w678	w851	w683	w1051	w782	w878	w830	w1045	w782
HLA class I (0-8)	3	5	3	7	3	6	8	0	0	4	0	5
CD8 Infiltration Score (0-5)	2	2	2	2	1	3	3	2	0	0	0	1
Corresponding CD8 density (cells/mm^2)	180-433	180-433	180-433	180-433	1-179	434-582	434-582	180-433	0	0	0	1-179
CD4 cells/mm^2	30	21	18.7	38.3	6	42.3	70	110.7	0	3	12	0.3
FoxP3 cells/mm^2	4.7	13	2.7	22	2.7	9	>50	6.7	0	.67	1.7	34.7
CM2B4 (0-8) (Large T-Ag)	8	8	8	8	7	8	8	8	8	8	8	8

Figure S3: T cell infiltrate of tumors characterized by TCR repertoire and IHC. (A) Tumors from 9 patients were analyzed for TCRβ repertoire and stained for HLA-I, CD8, CD4, and FoxP3. Due to low DNA yield from patient w782's primary tumor, the patient's nodal recurrence was also characterized. Tumors contained between 16 and 41,645 unique TCRs. Intratumoral CD8+ infiltration was categorized on a 0-5 scale as previously described [14]. CD8+ cells infiltrated tumors more frequently than CD4+ or FoxP3+ cells in most tumors, suggesting that most TCRs are likely from CD8+ T cells. Primary tumors = Black and white bars; lymph nodes = blue; metastasis = red. **(B)** Patients were separated *a priori* into those with many T cells (≥0.8 T cells/ng tumor DNA, n=7) or few T cells (<0.3 T cells/ng tumor DNA, n=3). There is no survival difference among patients based on their general immune infiltrate (p=0.59 by log-rank test).

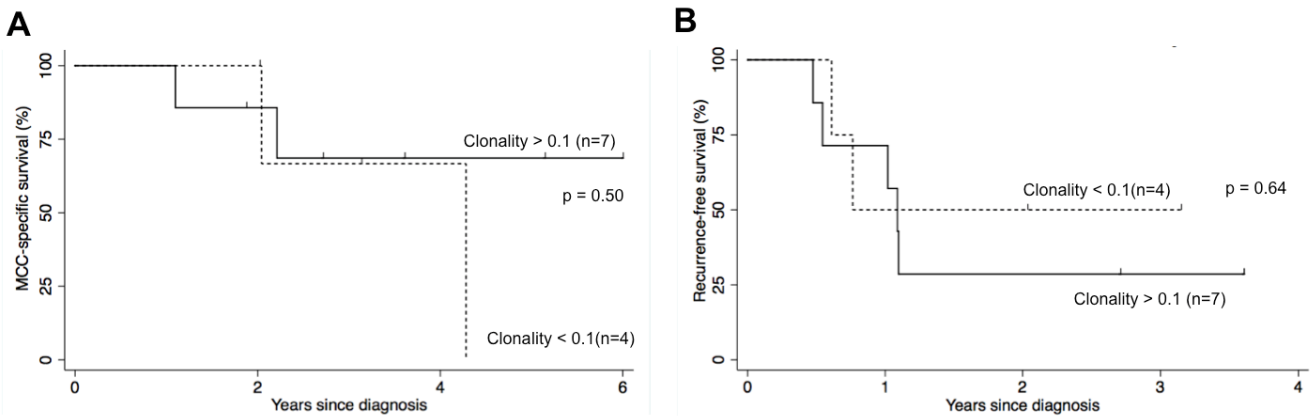


Figure S4: Clonality of the T cell repertoire within tumors of MCC patients does not correlate with disease outcome. Patients were binned by whether their tumors had high (>0.1, n=6) or low (<0.1, n=3) clonality. MCC-specific survival **(A)** or recurrence-free survival **(B)** between the two groups of patients was not significantly different by univariate analysis (p=0.50 or p=0.64 by log-rank test).

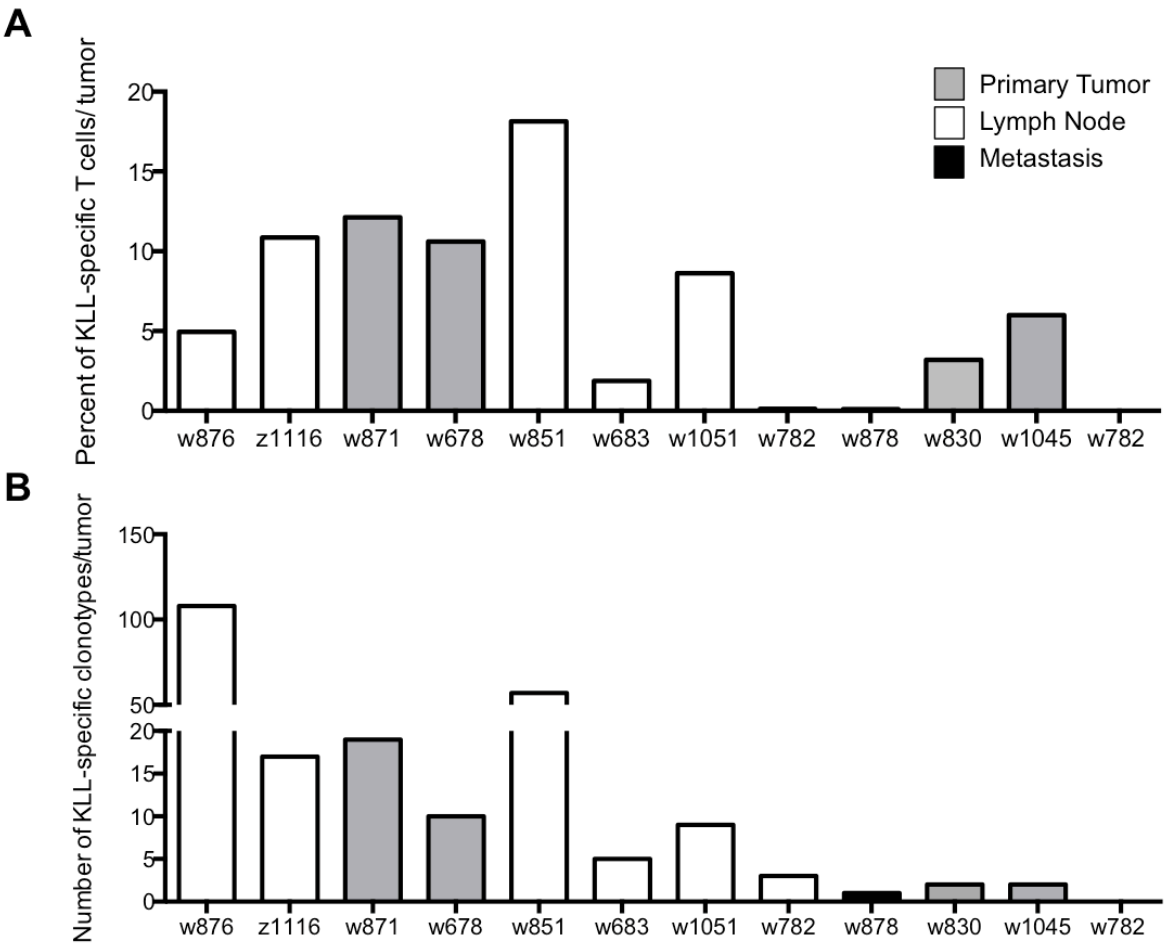


Figure S5: Percentage and number of KLL-specific clonotypes amid tumors. (A) KLL-specific T cells constituted between 0-18% of the T cell repertoire of each tumor based on number of genomes sequenced. **(B)** Tumors contained between 0-108 unique KLL-specific clonotypes.

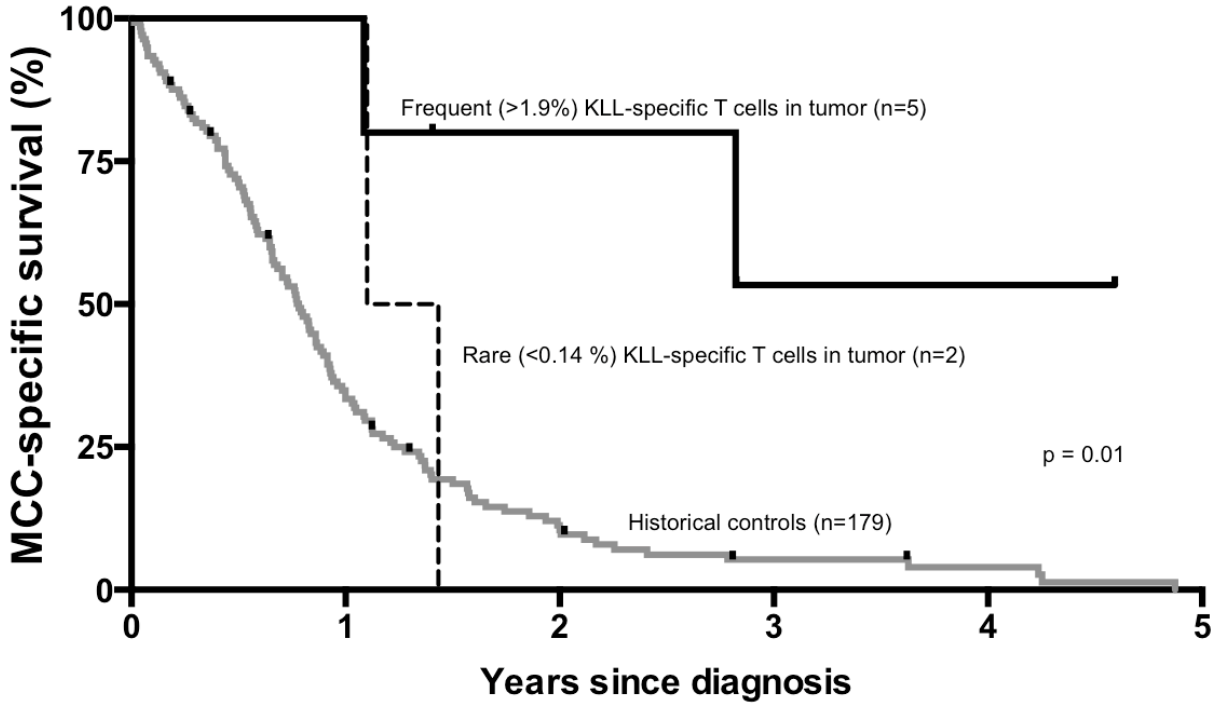


Figure S6: Patients with increased infiltration of KLL-specific T cells have increased survival after developing metastatic disease (p=0.15 by log-rank test), and is significant when compared to survival of a historical cohort of n=179 patients who developed metastatic disease treated at our institution (p=0.01 by log-rank test).

Table S3: List of all TCRβ clonotypes resolved from HLA-A*02:01/KLL-tetramer sorted T cells, annotated by patient

CDR3	TCRBV allele	TCRBJ allele		CDR3	TCRBV allele	TCRBJ allele
w678				**w782 cont'd**		
CAIRQFDANTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSPPSSGNTIYF	TCRBV18-01*01	TCRBJ01-03*01
CASSIIAGSSYNEQFF	TCRBV19-01	TCRBJ02-01*01		CASSVRVQQRKNIQYF	TCRBV21-01*01	TCRBJ02-04*01
CASSSGNPSTDTQYF	TCRBV10-02*01	TCRBJ02-03*01		CAIRTLDMNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSGGLLHVLDEQYF	TCRBV21-01*01	TCRBJ02-07*01		CSARPGQGAYNSPLHF	TCRBV20	TCRBJ01-06*01
CATTWRRYYEQYF	TCRBV06-07*01	TCRBJ02-07*01		CASSLYREETQYF	TCRBV07-07*01	TCRBJ02-05*01
w683				**w830**		
CASRSQNYYGYTF*	TCRBV06-05*01	TCRBJ01-02*01		CASSIMLYSNQPQHF	TCRBV19-01	TCRBJ01-05*01
CASSILLVPIATNEKLFF	TCRBV19-01	TCRBJ01-04*01		CAIRARDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASRSQNYYGYTF*	TCRBV06-06	TCRBJ01-02*01		CASSILGASNQPQHF*	TCRBV19-01	TCRBJ01-05*01
CASRSQNYYGYTF*	TCRBV06-01*01	TCRBJ01-02*01		CASSLAGFRFF	TCRBV12	TCRBJ02-01*01
CASRSQNYYGYTF*	TCRBV06	TCRBJ01-02*01		CASSLTGLAGTDTQYF	TCRBV07-03*01	TCRBJ02-03*01
CASRSQNYYGYTF*	TCRBV06	TCRBJ01-02*01		CAIRKQDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSRALATARKNIQYF	TCRBV21-01*01	TCRBJ02-04*01		CASSFPGAGSNTGELFF	TCRBV28-01*01	TCRBJ02-02*01
CASSLSMLQQRKNIQYF	TCRBV21-01*01	TCRBJ02-04*01		CASSLVIATQIRTEAFF	TCRBV21-01*01	TCRBJ01-01*01
CASRSQNYYGYTF*	TCRBV06-08*01	TCRBJ01-02*01		CASSILGASNQPQHF*	TCRBV19-01	TCRBJ01-05*01
CASRSQNYYGYTF*	TCRBV06-09*01	TCRBJ01-02*01		CASRGLLAQQSRANVLTF	TCRBV21-01*01	TCRBJ02-06*01
CASRSQNYYGYTF*	TCRBV06-07*01	TCRBJ01-02*01		CASRHWLLQHARNTIYF	TCRBV21-01*01	TCRBJ01-03*01
CASRSQNYYGYTF*	TCRBV06-04	TCRBJ01-02*01		CASSNPQRIAQSRANVLTF	TCRBV10-01	TCRBJ02-06*01
CASRSQNYYGYTF*	TCRBV06	TCRBJ01-02*01		CPGSRYGSEQSRANVLTF	TCRBV22-01*01	TCRBJ02-06*01
CASSSQNYYGYTF	TCRBV06-05*01	TCRBJ01-02*01		CASSILLYSNQPQHF	TCRBV19-01	TCRBJ01-05*01
CASSVALLQHARNTIYF	TCRBV21-01*01	TCRBJ01-03*01		CASSWSVLQHARNTIYF	TCRBV21-01*01	TCRBJ01-03*01
CASRAKLATLRTEAFF	TCRBV21-01*01	TCRBJ01-01*01		CASSLGWGDTEAFF	TCRBV12	TCRBJ01-01*01
CASRSQNYYGYTF*	TCRBV10-03*01	TCRBJ01-02*01		CASSLTGLAGTDTQYF	TCRBV07-03*01	TCRBJ02-03*01
CASRSQNYYGYTF*	TCRBV06	TCRBJ01-02*01				
CASRSQNYYGYTF*	TCRBV06	TCRBJ01-02*01		**w851**		
CASKTGGREKLFF	TCRBV28-01*01	TCRBJ01-04*01		CASSILSNSYNEQFF	TCRBV19-01	TCRBJ02-01*01
CASKKLDRPAPNSPLHF	TCRBV03	TCRBJ01-06*01		CASRRAPGGGLYNEQFF	TCRBV03	TCRBJ02-01*01
CASSEFLRGADYGYTF	TCRBV25-01*01	TCRBJ01-02*01		CAIRTLDMNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSLVGGRDEQYF	TCRBV09-01	TCRBJ02-07*01		CASSLSRGLLNGYTF	TCRBV27-01*01	TCRBJ01-02*01
				CASSLVGGRDGYTF	TCRBV12	TCRBJ01-02*01
w750				CASSQFWAGGIYEQYF	TCRBV03	TCRBJ02-07*01
CAIRDSNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSQVGETQYF	TCRBV04-01*01	TCRBJ02-05*01
CSARDLLAGTNTGELFF	TCRBV20	TCRBJ02-02*01		CASSYQGEEETQYF	TCRBV06-05*01	TCRBJ02-05*01
CAIRLADQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CATSSDRGGLQETQYF	TCRBV15-01*01	TCRBJ02-05*01
CASRDIGSGPQHF	TCRBV10-02*01	TCRBJ01-05*01		CASRHNVLQHARNTIYF	TCRBV21-01*01	TCRBJ01-03*01
CASRDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSGRLQQSRANVLTF	TCRBV21-01*01	TCRBJ02-06*01
CAIRIRDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSYPYGGGQNEQFF	TCRBV06-05*01	TCRBJ02-01*01
CASRTIFATVMQDTQYF	TCRBV21-01*01	TCRBJ02-03*01		CARGPTGGYTF	TCRBV02-01*01	TCRBJ01-02*01
CAIRTRDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSPRAGVDYGYTF	TCRBV18-01*01	TCRBJ01-02*01
CASSRLQQRKNIQYF	TCRBV21-01*01	TCRBJ02-04*01		CASSLVRDSYNEQFF	TCRBV07-02*01	TCRBJ02-01*01
CASSIMVYSYNEQFF	TCRBV19-01	TCRBJ02-01*01		CASSGGRVNEKLFF	TCRBV19-01	TCRBJ01-04*01
CAIREGDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSLGGNTGELFF	TCRBV27-01*01	TCRBJ02-02*01
CASSDFNPSTDTQYF	TCRBV06-01*01	TCRBJ02-03*01		CASSEWGGTQPQHF	TCRBV06-01*01	TCRBJ01-05*01
CASSRGSVSDEQYF	TCRBV19-01	TCRBJ02-07*01		CATSGTGRWETQYF	TCRBV15-01*01	TCRBJ02-05*01
CASSDRDLYGYTF	TCRBV19-01	TCRBJ01-02*01		CASSLARGPGNTIYF	TCRBV07-06*01	TCRBJ01-03*01
CASSIAAGDAYGYTF	TCRBV19-01	TCRBJ01-02*01		CASRITMGQPQHF	TCRBV19-01	TCRBJ01-05*01
CASSPRGDTEAFF	TCRBV10-01	TCRBJ01-01*01		CASSDRVAGNEQFF	TCRBV06-05*01	TCRBJ02-01*01
CASSFGSEQYF	TCRBV05-04*01	TCRBJ02-07*01		CASSLTSGVTEAFF	TCRBV07-09	TCRBJ01-01*01
CASSWELTNEQYF	TCRBV05-04*01	TCRBJ02-07*01		CASSLSPELHGYTF	TCRBV27-01*01	TCRBJ01-02*01
CASNRGSTQSRANVLTF	TCRBV05-02*01	TCRBJ02-06*01		CATSRDSGGLDGDTQYF	TCRBV15-01*01	TCRBJ02-03*01
CASSWRVQPQHF	TCRBV28-01*01	TCRBJ01-05*01		CASSPGEWGSETQYF	TCRBV03	TCRBJ02-05*01
CASSQSIADNYGYTF	TCRBV16-01	TCRBJ01-02*01		CASSFGGGANEQFF	TCRBV13-01*01	TCRBJ02-01*01
CASSLSGQPQHF	TCRBV27-01*01	TCRBJ01-05*01		CASTPGGLPKNIQYF	TCRBV11-01*01	TCRBJ02-04*01
				CASSATGTGDLEQFF	TCRBV07-02*01	TCRBJ02-01*01
w782				CASSWGYDSYNEQFF	TCRBV05-06*01	TCRBJ02-01*01
CASSILGYSNQPQHF	TCRBV19-01	TCRBJ01-05*01		CASSQETGEGNSPLHF	TCRBV04-02*01	TCRBJ01-06*01
CAIRDSNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASRLTDRGRVGEKLFF	TCRBV07-09	TCRBJ01-04*01
CAIRAGDSNTGELFF	TCRBV10-03*01	TCRBJ02-02*01		CASSILSNSYNEQFF	TCRBV19-01	TCRBJ02-01*01
CASREGAAYNEQFF**	TCRBV06-01*01	TCRBJ02-01*01		CASSAGTAAGNTIYF	TCRBV07-06*01	TCRBJ01-03*01
CASREGAAYNEQFF**	TCRBV06	TCRBJ02-01*01		CASSGVKRSHKSRANVLTF	TCRBV10-01	TCRBJ02-06*01
CATSDPLAASYEQYF	TCRBV24	TCRBJ02-07*01		CASSGYHDGFSEQYF	TCRBV06-01*01	TCRBJ02-07*01

CDR3	TCRBV allele	TCRBJ allele
w851 cont'd		
CASSLQGAGQPQHF	TCRBV19-01	TCRBJ01-05*01
CADGRGDEQYF	TCRBV02-01*01	TCRBJ02-07*01
CASSPVGGDQPQHF	TCRBV07-09	TCRBJ01-05*01
CASSIGRTYYGYTF	TCRBV19-01	TCRBJ01-02*01
CAYGAGGPNTEAFF	TCRBV05-08*01	TCRBJ01-01*01
CASNIYSQPQHF	TCRBV19-01	TCRBJ01-05*01
CASSLEGDTEAFF	TCRBV05-05*01	TCRBJ01-01*01
CASSETDRGLAYEQYV	TCRBV06-01*01	TCRBJ02-07*01
CSARDRVGNTIYF	TCRBV20	TCRBJ01-03*01
CASSYFPGVEAFF	TCRBV06-05*01	TCRBJ01-01*01
CASSEGQGNSPLHF	TCRBV09-01	TCRBJ01-06*01
CASQTGFYNEQFF	TCRBV06-05*01	TCRBJ02-01*01
CASKTSGFPDTQYF	TCRBV02-01*01	TCRBJ02-03*01
CASSLSRGDSNQPQHF	TCRBV27-01*01	TCRBJ01-05*01
CASRESNTEAFF	TCRBV27-01*01	TCRBJ01-01*01
CASSEGQSYEQYF	TCRBV05-06*01	TCRBJ02-07*01
CASSSGTPSTDTQYF	TCRBV06-06	TCRBJ02-03*01
CASRPDIPLGETQYF	TCRBV06-05*01	TCRBJ02-05*01
CASSILSNSYNEQFF	TCRBV19-01	TCRBJ02-01*01
CASKKLDRPAPNSPLHF	TCRBV03	TCRBJ01-06*01
CASRRAPGGGLYNEQFS	TCRBV03	TCRBJ02
CASSYQGEEETQYF	TCRBV06	TCRBJ02-05*01
w871		
CASSSGTPSTDTQYF	TCRBV06-06	TCRBJ02-03*01
CAINNRDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASTQSNTGELFF	TCRBV10-02*01	TCRBJ02-02*01
CASSETPDMNTEAFF	TCRBV06-01*01	TCRBJ01-01*01
CASSSGTPSTDTQYF*	TCRBV06-05*01	TCRBJ02-03*01
CASSSGTPSTDTQYF*	TCRBV06	TCRBJ02-03*01
CASTDSNTGELFF	TCRBV10-02*01	TCRBJ02-02*01
CASSSGTPSTDTQYF*	TCRBV06-05*01	TCRBJ02-03*01
CASSSGTPSTDTQYF*	TCRBV06-09*01	TCRBJ02-03*01
CASSSGTPSTDTQYF*	TCRBV06-09*01	TCRBJ02-03*01
CASSLGVAGGSSYNEQFF	TCRBV13-01*01	TCRBJ02-01*01
CASSYSTGVPEKLFF	TCRBV06-05*01	TCRBJ01-04*01
CASSWYLATHSDNEQFF	TCRBV21-01*01	TCRBJ02-01*01
CASTGGLADTQYF	TCRBV19-01	TCRBJ02-03*01
CASSSCMDIYKSRANVLTF	TCRBV18-01*01	TCRBJ02-06*01
CASRRTSGGRTDTQYF	TCRBV06	TCRBJ02-03*01
CASSSGTPSTDTQYF*	TCRBV06-08*01	TCRBJ02-03*01
CASSSGTPSTDTQYF*	TCRBV06-06	TCRBJ02-03*01
w876 (PBMC)		
CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CAIRRQDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CAIHEGDSNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASRGQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSQLRTGDEYEQYF	TCRBV16-01	TCRBJ02-07*01
CATRDINTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRAGASYNEQFF	TCRBV28-01*01	TCRBJ02-01*01
CAISARDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSFGSGTKDTQYF	TCRBV12	TCRBJ02-03*01
CASRGSIATRYNEKLFF	TCRBV21-01*01	TCRBJ01-04*01
CASSDTPDLNTEAFF*	TCRBV06-01*01	TCRBJ01-01*01
CASSLAGLAGTDTQYF	TCRBV07-02*01	TCRBJ02-03*01
CASSSRTKAYEQYF	TCRBV13-01*01	TCRBJ02-07*01
CARTESRQSRANVLTF	TCRBV07-05*01	TCRBJ02-06*01
CASSVEDYTGELFF*	TCRBV09-01	TCRBJ02-02*01
CASRDRREQFF	TCRBV21-01*01	TCRBJ02-01*01
CASRRVLAYRKTYGYTF	TCRBV21-01*01	TCRBJ01-02*01
CASRRCIATHTHNSPLHF	TCRBV21-01*01	TCRBJ01-06*01
CAISADNCIQSRANVLTF	TCRBV10-03*01	TCRBJ02-06*01
CASSGQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASRGDIGYRKTYGYTF	TCRBV21-01*01	TCRBJ01-02*01
CASSILSSSNQPQHF	TCRBV19-01	TCRBJ01-05*01
CASTLGNPSTDTQYF	TCRBV06-06	TCRBJ02-03*01
CASSSGTSGGLNYNEQFF	TCRBV13-01*01	TCRBJ02-01*01
CASSSGTSGGLTYNEQFF	TCRBV13-01*01	TCRBJ02-01*01
CASSTLSGTHNEQFF	TCRBV19-01	TCRBJ02-01*01
CASSAEVTNHQSRANVLTF	TCRBV19-01	TCRBJ02-06*01
CASSDTPDLNTEAFF*	TCRBV06	TCRBJ01-01*01
CASSYSTGVPEKLFF	TCRBV06-05*01	TCRBJ01-04*01
w876 (TIL)		
CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CAIRAGASYNEQFF*	TCRBV28-01*01	TCRBJ02-01*01
CASRGQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIHEGDSNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAISARDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRRQDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRGQDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CATRDINTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASSQLRTGDEYEQYF	TCRBV16-01	TCRBJ02-07*01
CASSDTPDLNTEAFF*	TCRBV06-01*01	TCRBJ01-01*01
CASSFGSGTKDTQYF*	TCRBV12	TCRBJ02-03*01
CAS SSRTKAYEQYF	TCRBV13-01*01	TCRBJ02-07*01
CASSLIAGLSYEQYF	TCRBV07-08*01	TCRBJ02-07*01
CASSLAGLAGTDTQYF	TCRBV07-02*01	TCRBJ02-03*01
CASTLGNPSTDTQYF*	TCRBV06-06	TCRBJ02-03*01
CASSGQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASSVEDYTGELFF*	TCRBV09-01	TCRBJ02-02*01
CASSIQLFVRTEAFF*	TCRBV19-01	TCRBJ01-01*01
CASRASNTYGYTF*	TCRBV06-05*01	TCRBJ01-02*01
CASSIIAYSNQPQHF	TCRBV19-01	TCRBJ01-05*01
CASRSQLAVLNEQFF	TCRBV19-01	TCRBJ02-01*01
CASSTLSGTHNEQFF	TCRBV19-01	TCRBJ02-01*01
CASSILSSSNQPQHF	TCRBV19-01	TCRBJ01-05*01
CASSLAGDRYF	TCRBV12	TCRBJ01-06*01
CCASSFGTSGGTTYNEQFF*	TCRBV13-01*01	TCRBJ02-01*01
CASSPWDEQFF	TCRBV12	TCRBJ02-01*01
CASRGGSSYNEQFF	TCRBV28-01*01	TCRBJ02-01*01
CASSSGTSGGLTYNEQFF	TCRBV13-01*01	TCRBJ02-01*01
CASSYQIGLSYEQYF*	TCRBV06-06	TCRBJ02-07*01
CASSEFAGQETQYF	TCRBV02-01*01	TCRBJ02-05*01
CASSSGTSGGLNYNEQFF	TCRBV13-01*01	TCRBJ02-01*01
CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASSVLNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIHEGDSNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASSDTPDLNTEAFF*	TCRBV06	TCRBJ01-01*01
CAIRRQDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASRGQNTGELFF*	TCRBV10	TCRBJ02-02*01
CAIRGQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASRASNTYGYTF*	TCRBV06-06	TCRBJ01-02*01
CASSSRTKAYEQYF*	TCRBV13-01*01	TCRBJ02-07*01
CASSDTPDLNTEAFF*	TCRBV06-09*01	TCRBJ01-01*01
CASSDTPDLNTEAFF*	TCRBV06-08*01	TCRBJ01-01*01
CASSVEDYTGELFF*	TCRBV09-01	TCRBJ02-02*01
CASTLGNPSTDTQYF*	TCRBV06-05*01	TCRBJ02-03*01
CASRASNTYGYTF*	TCRBV06	TCRBJ01-02*01
CASRTVVLHWHHQPQHF	TCRBV21-01*01	TCRBJ01-05*01
CAIRTGSAYNEQFF	TCRBV28-01*01	TCRBJ02-01*01
CAISARDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASSDTPDLNTEAFF*	TCRBV10-03*01	TCRBJ01-01*01
CSALPVTGAFQETQYF	TCRBV20	TCRBJ02-05*01
CASSVLNTGELFF	TCRBV10-01	TCRBJ02-02*01
CAIRGQDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASRASNTYGYTF*	TCRBV06-01*01	TCRBJ01-02*01

CDR3	TCRBV allele	TCRBJ allele		CDR3	TCRBV allele	TCRBJ allele
w876 (TIL) cont'd				**w876 (TIL) cont'd**		
CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CASRDINSGELFF	TCRBV10-03*01	TCRBJ02-02*01
CARSVLNTGELFF	TCRBV10-02*01	TCRBJ02-02*01		CASSVLNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRRQDQNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01		CASTLGNPSTDTQYF*	TCRBV10-03*01	TCRBJ02-03*01
CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CACSVLNTGELFF	TCRBV10-02*01	TCRBJ02-02*01
CAIHEGDSNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01		CAIHEGDSNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASSVLNTGELFF*	TCRBV03	TCRBJ02-02*01		CAIHEGDSNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASSPTGAVSYEQYF	TCRBV12	TCRBJ02-07*01		CAIRAGASYNEQFF*	TCRBV28-01*01	TCRBJ02-01*01
CSARAPTGTGNTGELFF	TCRBV20	TCRBJ02-02*01		CAIRAVASYNEQFF	TCRBV28-01*01	TCRBJ02-01*01
CATRDINTGELFF*	TCRBV10	TCRBJ02-02*01		CAIRGQDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRRQDQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CAIRRQDHNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CAISARDQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CAIRRQDQNNGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASRGQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CASRASNTYGYTF*	TCRBV10-03*01	TCRBJ01-02*01
CASRGQNTGELFF*	unresolved	TCRBJ02-02*01		CASRGQDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CAIRGQDQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CASSLIAGLSYEQYF*	TCRBV07-04*01	TCRBJ02-07*01
CAIRRQDQNTGELFF*	TCRBV06-06	TCRBJ02-02*01		CAIHEGDSNTGELFF*	TCRBV06-06	TCRBJ02-02*01
CASSGQNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CASSQLRTGDEYEQYF*	TCRBV16-01	TCRBJ02-07*01
CAIRGQDQNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01		CASSSRTKAYEQYF*	TCRBV05-02*01	TCRBJ02-07*01
CASSSRTKAYEQYF*	TCRBV02-01*01	TCRBJ02-07*01		CAIRRQDQNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01
CASSSRTKAYEQYF*	TCRBV27-01*01	TCRBJ02-07*01		CAIRRQDQNTGELFF*	unresolved	TCRBJ02-02*01
CASTLGNPSTDTQYF*	TCRBV06-09*01	TCRBJ02-03*01		CAISARDQNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01
CATRDINTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CAISARDQNTGELFF*	TCRBV06	TCRBJ02-02*01
CASSDRPRIAQSRANVLTF	TCRBV10-01	TCRBJ02-06*01		CAISDTPDLNTEAFF	TCRBV06-01*01	TCRBJ01-01*01
CASRRCIATTARNTIYF	TCRBV21-01*01	TCRBJ01-03*01		CANSSRTKAYEQYF	TCRBV13-01*01	TCRBJ02-07*01
CASSESNTLVGFF	TCRBV10-02*01	TCRBJ02-01*01		CASRASNTYGYTF*	TCRBV06-08*01	TCRBJ01-02*01
CPGRRARKRTSRANVLTF	TCRBV22-01*01	TCRBJ02-06*01		CASSDTPDLNTEAFF*	TCRBV03	TCRBJ01-01*01
CASSLFSVYTQFF	TCRBV12	TCRBJ02-01*01		CASSDTPDLNTEAFF*	TCRBV06-01*01	TCRBJ01-01*01
CASSLGVSGGMTYNEQFF	TCRBV13-01*01	TCRBJ02-01*01		CASSDTPDLNTEAFF*	TCRBV06-01*01	TCRBJ01-01*01
CPGSRLGSEQSRANVLTF	TCRBV22-01*01	TCRBJ02-06*01		CASSDTPDLNTEAFF*	TCRBV06-01*01	TCRBJ01-01*01
CASSVLNTGELFF*	TCRBV10-01	TCRBJ02-02*01		CASSFGSGTKDTQYF*	TCRBV03	TCRBJ02-03*01
CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CASSFGSGTKDTQYF*	TCRBV03	TCRBJ02-03*01
CAIRGQDQNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01		CASSFGSGTKDTQYF*	TCRBV07-04*01	TCRBJ02-03*01
CASSLAGLAGTDTQYF*	TCRBV11-02*02	TCRBJ02-03*01		CASSFGSGTKDTQYF*	TCRBV12	TCRBJ02-03*01
CASSVLNTGELFF*	TCRBV06-06	TCRBJ02-02*01		CASSLAGLAGTDTQYF*	TCRBV07-06*01	TCRBJ02-03*01
CAIHEGDSNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01		CASSLAGLAGTDTQYF*	TCRBV07-03*01	TCRBJ02-03*01
CAIHEGDSNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01		CASSLIAGLSYEQYF*	TCRBV11-02*02	TCRBJ02-07*01
CASRASNTYGYTF*	TCRBV06-09*01	TCRBJ01-02*01		CASSLIAGLSYEQYF*	TCRBV07-01*01	TCRBJ02-07*01
CASRASNTYGYTF*	TCRBV06	TCRBJ01-02*01		CASSLIAGLSYEQYF*	TCRBV07-06*01	TCRBJ02-07*01
CASRGQNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01		CASSQLRTGDEYEQYF*	TCRBV13-01*01	TCRBJ02-07*01
CASRGQNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01		CASSSRTKAYEQYF*	TCRBV03	TCRBJ02-07*01
CASRGQNTGELFF*	TCRBV06-06	TCRBJ02-02*01		CASSSRTKAYEQYF*	TCRBV03	TCRBJ02-07*01
CASSDTPDLNTEAFF*	TCRBV06-01*01	TCRBJ01-01*01		CASSSRTKAYEQYF*	TCRBV02-01*01	TCRBJ02-07*01
CCASSFGTSGGTTYNEQFF*	TCRBV13-01*01	TCRBJ02-01*01		CASSSRTKAYEQYF*	TCRBV02-01*01	TCRBJ02-07*01
CASSIQLFVRTEAFF*	TCRBV19-01	TCRBJ01-01*01		CASSSRTKAYEQYF*	TCRBV13-01*01	TCRBJ02-07*01
CASSLAGLAGTDTQYF*	TCRBV07-09	TCRBJ02-03*01		CASSSRTKAYEQYF*	TCRBV13-01*01	TCRBJ02-07*01
CASSLIAGLSYEQYF*	TCRBV07-03*01	TCRBJ02-07*01		CASSVEDYTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASSRYGQGWEQYF	TCRBV27-01*01	TCRBJ02-07*01		CASSVLNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01
CASSSRTKAYEQYF*	TCRBV13-01*01	TCRBJ02-07*01		CASSVLNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01
CASSSRTKAYEQYF*	TCRBV13-01*01	TCRBJ02-07*01		CASSVLNTGELFF*	TCRBV06-09*01	TCRBJ02-02*01
CASSVEDYTGELFF*	TCRBV03	TCRBJ02-02*01		CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASSVLNTGELFF*	TCRBV09-01	TCRBJ02-02*01		CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASSVLNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01		CASSVLNTGELFF*	TCRBV10-02*01	TCRBJ02-02*01
CASSVLNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01		CASSYQIGLSYEQYF*	TCRBV06	TCRBJ02-07*01
CASSYQIGLSYEQYF*	TCRBV06-05*01	TCRBJ02-07*01		CASTLGNPSTDTQYF*	TCRBV06	TCRBJ02-03*01
CASREGYSNQPQHF	TCRBV19-01	TCRBJ01-05*01		CATRDINTGELFF*	TCRBV06-01*01	TCRBJ02-02*01
CASSGRDRGSEKLFF	TCRBV19-01	TCRBJ01-04*01				
CASSGQVATHARNTIYF	TCRBV21-01*01	TCRBJ01-03*01		**w878**		
CASSHGRLNEKLFF	TCRBV13-01*01	TCRBJ01-04*01		CASRGGASYNEQFF	TCRBV28-01*01	TCRBJ02-01*01
CATSHSTVGYGYTF	TCRBV10-03*01	TCRBJ01-02*01		CASSILLFSGNTIYF	TCRBV19-01	TCRBJ01-03*01
CASSFDSKGSNTGELFF	TCRBV28-01*01	TCRBJ02-02*01		CAIRSRDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSLIIGRDPYEQYF	TCRBV07-09	TCRBJ02-07*01		CASSQDARRSGNTIYF	TCRBV14-01*01	TCRBJ01-03*01
CASSLVPSGSPVSAGELFF	TCRBV11-02*02	TCRBJ02-02*01		CASSIQEGYSEQYF	TCRBV19-01	TCRBJ02-07*01
CASSLWVAGYNEQFF	TCRBV07-09	TCRBJ02-01*01		CASSPALATTSRANVLTF	TCRBV21-01*01	TCRBJ02-06*01
CSARLANSYEQYF	TCRBV20	TCRBJ02-07*01		CASRTSNTYGYTF	TCRBV06-05*01	TCRBJ01-02*01
CAISARDQNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01		CAIRAADQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01

CDR3	TCRBV allele	TCRBJ allele
w878 cont'd		
CASRQFLATPSDNEQFF	TCRBV21-01*01	TCRBJ02-01*01
CASSLLRTSQETQYF	TCRBV12	TCRBJ02-05*01
CASSIQEGYSEQYF	TCRBV19-01	TCRBJ02-05*01
YASSDKSLGGVDTGELFF	TCRBV26-01*01	TCRBJ01-03*01
w1045		
CASRTGSSYNEQFF	TCRBV28-01*01	TCRBJ02-01*01
CASSTGEPGVYGYTF	TCRBV06-05*01	TCRBJ01-02*01
CASTPGAGLKNEQFF	TCRBV06-05*01	TCRBJ02-01*01
CASSTGEPGVYGYTF	TCRBV06-01*01	TCRBJ01-02*01
CASSLDWRGNTIYF	TCRBV07-02*01	TCRBJ01-03*01
CAIRAYGQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSIELRSYEQYF	TCRBV19-01	TCRBJ02-07*01
CASTTGEGYEQYF	TCRBV06-05*01	TCRBJ02-07*01
CASSSGASLLNEQFF	TCRBV06-05*01	TCRBJ02-01*01
w1051		
CSARTGYNEQFF	TCRBV20	TCRBJ02-01*01
CASILIAGGYNEQFF	TCRBV02-01*01	TCRBJ02-01*01
CASILIAGAYNEQFF	TCRBV02-01*01	TCRBJ02-01*01
CASSPEGSGGYTF	TCRBV18-01*01	TCRBJ01-02*01
CASRCLVLQQSRANVLTF	TCRBV21-01*01	TCRBJ02-06*01
CASSADRGGWSGNQPQHF	TCRBV12	TCRBJ01-05*01
w1116 (PBMC)		
CAIRTLDMNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSLNIAHHSDNEQFF	TCRBV21-01*01	TCRBJ02-01*01
CASKRLAGEGTGELFF	TCRBV06	TCRBJ02-02*01
CAISTLDMNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CAIRTLDMNTGELFF	unresolved	TCRBJ02-02*01
CASSSSTEILWLHL	TCRBV28-01*01	TCRBJ01-02*01
w1116 (TIL)		
CAIRTLDMNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CASSGPDGDNEQFF	TCRBV09-01	TCRBJ02-01*01
CAIRTLDMNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CASSYPDVYEQYF*	TCRBV06	TCRBJ02-07*01
CAIRTLDMNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRIRDQNTGELFF	TCRBV10-03*01	TCRBJ02-02*01
CAIRTLDMNTGELFF*	TCRBV06-05*01	TCRBJ02-02*01
CASSYPDVYEQYF*	TCRBV06	TCRBJ02-05*01
CASSETGTWDEQYF	TCRBV10-02*01	TCRBJ02-07*01
CAIRTLDMNTGELFF*	TCRBV10-03*01	TCRBJ02-02*01
CAIRTLDMNTGELLF	TCRBV10-03*01	TCRBJ02-02*01
CAIRTLDMNTGELFF*	TCRBV06-06	TCRBJ02-02*01
CASSSSTESYGYTF	TCRBV28-01*01	TCRBJ01-02*01
CAIRTLDMNTGELFF*	TCRBV06-01*01	TCRBJ02-02*01
CASSGPDGDNEQFF	TCRBV09-01	TCRBJ02-01*01
CASSERHLHARNTIYF	TCRBV03	TCRBJ01-03*01
CASRSLIATLLDEQYF	TCRBV21-01*01	TCRBJ02-07*01
CASSSTLKSQSRANVLTF	TCRBV19-01	TCRBJ02-06*01
CAISEPSGAQHF	TCRBV10-03*01	TCRBJ01-05*01
CASSEGKTKSQSRANVLTF	TCRBV19-01	TCRBJ02-06*01
CASSLGNTEAFF	TCRBV11-02*02	TCRBJ01-01*01
CASSLVSSGGEAFF	TCRBV07-09	TCRBJ01-01*01
CAIRTLDMNTGDLFF	TCRBV10-03*01	TCRBJ02-02*01
CASKKLDRPAPNSPLHF	TCRBV03	TCRBJ01-06*01
CASSGPDGGNEQFF*	TCRBV09-01	TCRBJ02-01*01
CASSGPDGGNEQFF*	TCRBV09-01	TCRBJ02-01*01
CASSSQRKSYGYTF	TCRBV28-01*01	TCRBJ01-02*01
CASSSSRKSYGYTF	TCRBV28-01*01	TCRBJ01-02*01
CATSDPLAASYEQYF	TCRBV24	TCRBJ02-07*01

*denotes non-unique CDR3s within a patient, encoded by a unique *TRB* nucleotide sequence and/or unique TCRBV or TCRBJ.

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Clinical Cancer Research

Merkel Polyomavirus-Specific T Cells Fluctuate with Merkel Cell Carcinoma Burden and Express Therapeutically Targetable PD-1 and Tim-3 Exhaustion Markers

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Human Cancer Biology

Clinical Cancer Research

Merkel Polyomavirus-Specific T Cells Fluctuate with Merkel Cell Carcinoma Burden and Express Therapeutically Targetable PD-1 and Tim-3 Exhaustion Markers

Olga K. Afanasiev[1,2], Lola Yelistratova[1], Natalie Miller[1,2], Kotaro Nagase[8], Kelly Paulson[1,3], Jayasri G. Iyer[1], Dafina Ibrani[1], David M. Koelle[3,4,5,6,7], and Paul Nghiem[1,2,6]

Abstract

Purpose: The persistent expression of Merkel cell polyomavirus (MCPyV) oncoproteins in Merkel cell carcinoma (MCC) provides a unique opportunity to characterize immune evasion mechanisms in human cancer. We isolated MCPyV-specific T cells and determined their frequency and functional status.

Experimental Design: Multiparameter flow cytometry panels and HLA/peptide tetramers were used to identify and characterize T cells from tumors ($n = 7$) and blood ($n = 18$) of patients with MCC and control subjects ($n = 10$). PD-1 ligand (PD-L1) and CD8 expression within tumors were determined using mRNA profiling ($n = 35$) and immunohistochemistry ($n = 13$).

Results: MCPyV-specific CD8 T cells were detected directly *ex vivo* from the blood samples of 7 out of 11 (64%) patients with MCPyV-positive tumors. In contrast, 0 of 10 control subjects had detectable levels of these cells in their blood ($P < 0.01$). MCPyV-specific T cells in serial blood specimens increased with MCC disease progression and decreased with effective therapy. MCPyV-specific CD8 T cells and MCC-infiltrating lymphocytes expressed higher levels of therapeutically targetable PD-1 and Tim-3 inhibitory receptors compared with T cells specific to other human viruses ($P < 0.01$). PD-L1 was present in 9 of 13 (69%) MCCs and its expression was correlated with CD8-lymphocyte infiltration.

Conclusions: MCC-targeting T cells expand with tumor burden and express high levels of immune checkpoint receptors PD-1 and Tim-3. Reversal of these inhibitory pathways is therefore a promising therapeutic approach for this virus-driven cancer. *Clin Cancer Res; 19(19); 5351–60. ©2013 AACR.*

Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer with a disease-associated mortality three times that of malignant melanoma (~46% vs. 15%, respectively; ref. 1). MCC is increasingly common with an estimated 1,600 cases per year in the United States (2), and the reported incidence has more than tripled over the past 20 years (3). This increasing incidence is partly due to improved detection using a specific immunohistochemical marker, cytokeratin-20 (4), but may also be due to the higher prevalence of known risk factors for MCC: chronic T-cell immune suppression and the number of Caucasians more than 50 years of age with extensive prior sun exposure (5). Furthermore, the recent discovery of the Merkel cell polyomavirus (MCPyV) and its causal association with at least 80% of MCCs (6–8) has provided insight into MCC pathogenesis and underscores the importance of characterizing MCPyV-specific immune responses.

The necessary and persistent (7) expression of MCPyV T-antigen (T-Ag) oncoproteins in MCC tumors provides an opportunity to study antitumor immunity by assessing responses against a viral, tumor-specific antigen. Although the role of T cells is variable among different human cancers, multiple lines of evidence suggest that cellular immune function is unusually important for survival in MCC. We have previously shown that intratumoral CD8 lymphocyte infiltration (9) and lack of systemic immune suppression (10) are each significantly associated with improved survival. Furthermore, recent evidence suggests that patients with MCC have T cells that are specific for persistently expressed viral oncoproteins (11). In this study, we made use of an extensive collection of clinically annotated longitudinally collected blood specimens to track the frequency and function of MCPyV-specific CD8 T cells. It is hoped that characterizing the molecular pathways involved in the inhibition of MCPyV-specific T-cell responses may guide the design of rational therapies to overcome tumor immune escape.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Merkel cell carcinoma (MCC) is an aggressive skin cancer (46% 5-year disease-associated mortality) without available disease-specific therapies. Prior studies show a requirement for persistent expression of Merkel cell polyomavirus (MCPyV) oncoproteins and the frequent presence of virus-specific T cells in patients with MCC. It is thus likely that immune evasion mechanisms are important in the pathogenesis of this immunogenic cancer. In this report, we identify several immune-inhibitory pathways that are active in MCPyV-specific T cells in patients with MCC. These findings have implications for the use of existing and emerging agents that may augment immune responses in this virus-associated cancer.

To assess the functional state of MCC-targeting CD8 T cells, it was critical to determine the expression of physiologically relevant cell-surface markers directly *ex vivo* from tumors or blood. Key pathways examined included those associated with T-cell inhibition (programmed death 1, PD-1; T-cell immunoglobulin and mucin-domain, Tim-3; cytotoxic T-lymphocyte antigen 4, CTLA-4), costimulation, and activation (CD28, CD69, CD137). Many of these molecules are the targets of therapeutic agents that are FDA-approved (ipilimumab for CTLA-4) or are in clinical (PD-1, CD137, or 4-1BB; refs. 12, 13) or preclinical (Tim-3; refs. 14, 15) trials. We show that while MCPyV-specific T-cell frequency increases and decreases in parallel with disease burden, these cells display an exhausted phenotypic profile throughout the disease course. Importantly, this study identifies key inhibitory and activation pathways that may be suitable therapeutic targets for reversing T-cell dysfunction and promoting antitumor responses.

Materials and Methods

Human subjects and samples

This study was approved by the Fred Hutchinson Research Center Institutional Review Board and conducted according to Declaration of Helsinki principles. Informed consent was received from all participants. Blood was obtained from HLA-A*2402$^+$, HLA-A*2301$^+$, or HLA-A*0201$^+$ subjects based on HLA restriction of available tetramers. Tumors were obtained from medically necessary procedures. Tumor-MCPyV status was assessed by real-time PCR (RT-PCR) for MCPyV T-Ag immunohistochemistry (CM2B4 antibody, Santa Cruz Biotechnology) and/or T-Ag serology (9). Extent of disease was determined by clinical evaluation and staging by AJCC 7th edition guidelines.

T-cell analysis and flow cytometry

Virus-specific T-cell frequencies in blood were assessed directly *ex vivo* using tetramers indicated below. Tumor-infiltrating lymphocytes (TIL) were obtained from fresh MCC tumors that were minced and digested with 0.1 mg/mL DNAse-I, 0.4 mg/mL collagenase-IV, 0.1 mg/mL hyaluronidase (all from Worthington Biochemical) in serum-free RPMI for 3 hours at 37°C, then passed through a 70 μm nylon cell strainer. Isolated lymphocytes were incubated for 30 minutes at 37°C with allophycocyanin (APC)-conjugated tetramers specific for MCPyV (11), cytomegalovirus (CMV), or Epstein–Barr virus (EBV; HLA-A24/MCPyV.LT-92-101, A2/CMV.pp65.495-503, or A2/EBV.BMLF1.280-288, respectively). Fc receptor block (Miltenyi Biotec) was added for 10 minutes at 4°C, and cells were stained for 30 minutes at 4°C with: CD3-Qdot605 (Invitrogen), CD8-V500 (BD), PD-1-BrilliantViolet421 (BioLegend), Tim-3-PE (R&D Systems), CTLA-4-FITC (Cedarlane), CD28-ECD (Beckman Coulter), CD69-PeCy5.5 (Invitrogen), CD137-PeCy7 (BioLegend), or isotype control antibodies. Cells were washed and fixed. At least 2 million events were collected on FACSAriaII machine (BD) and analyzed using FlowJo (Tree Star, Inc). Mean fluorescence intensity for PD-1 was determined for PD-1–positive populations.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue was stained with anti-PD-L1 (clone 5H1) at BioPillar Laboratories using previously described methods (16) and scored in a four-tiered system according to staining intensity, as previously described (17): strong (grade 3), moderate (grade 2), weak (grade 1), or no (grade 0) expression, in comparison with external controls (tonsil). As previously described, grades 3 and 2 were grouped as high-expresser cases and grade 1 or 0 were defined as low-expresser cases (18). Tumor-infiltrating CD8 lymphocytes were scored as previously described (9). MCPyV T-antigen staining was done using CM2B4 (Santa Cruz; ref. 19) and/or Ab3 (8) antibodies.

mRNA profiling

mRNA profiling and analysis was carried out as previously described (9) and relevant expression data were extracted from the publically available GEO database (accession number GSE22396).

IFN-γ functional assays

CD8 cells were negatively selected (MACS Kit, Miltenyi Biotec), plated at 1 to 2 × 10^5 cells per well with 1.5 × 10^4 autologous peripheral blood mononuclear cells (PBMC) used as APC, and stimulated in duplicate wells with peptides specific for MCPyV (MCPyV.LT-92-101, 10 μg/mL), EBV (BMLF1.280-288, 10 μg/mL), or media (negative control). Blocking anti-Tim-3 (10 μg/mL, Biolegend) and anti-PD-1 (10 μg/mL, R&D Systems), or isotype control monoclonal antibodies (10 μg/mL) were added. For assays conducted directly *ex vivo*, cells were plated directly onto 96-well multiscreenIP plates (Millipore) precoated with anti-IFN-γ capture antibody (1-D1K; Mabtech). For cultured assays, cells were stimulated on day 0 as above in 96-well round bottom plates with fresh T cell medium and 20 U/mL IL-2 (Chiron Corporation), 20 ng/mL IL-7 (R&D Systems), and 10 ng/mL IL-15 (R&D Systems) added on days 2, 4, and 6. On day 7, cells were transferred to IFN-γ precoated ELISPOT plates and mitogens corresponding to the prior stimulation

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cycle were added. ELISPOT plates were developed after 20 hours, scanned with an enzyme-linked immunospot reader (AID), counted using EliSpot Reader software (AID), and verified for quality control. Representative experiments are shown, with each experiment conducted at least twice. Data are presented as net spot forming units (SFU), which is the average SFU of duplicate wells minus the average SFU in the negative control well. Experiments in patients whose cells failed to proliferate with culture or exhibited high background signal were not interpretable and were not included.

The intracellular IFN-γ assay was conducted as previously described (11) and is detailed in Supplementary Methods.

Statistical analysis

For quantitative comparisons, Fisher exact test, Wilcoxon rank sum test, or Student *t* test was conducted with Stata11 (StataCorp); $P < 0.05$ was considered as significant.

Results

CD8 T cells specific for MCPyV T-Ag are detectable in MCC patients, but not in control subjects

To investigate the prevalence of MCPyV-specific T cells found in the blood of patients with MCC and control subjects, we used an HLA-peptide tetramer (HLA-A24: MCPyV.LT.92-101) in a direct *ex vivo* screen of HLA-compatible PBMCs from first available blood draw. Viral oncoprotein-specific CD8 T cells were not detectable in blood from any of 10 HLA-A24 control subjects in this assay (detection sensitivity of ∼0.01% of CD8 T cells). In contrast, 64% of HLA-compatible patients with MCC (7/11; $P < 0.01$) had MCPyV-specific T cells in their blood (range: 0.03%–0.24% of CD8 T cells; Fig. 1A, Supplementary Fig. S1). Patients with detectable virus-specific T cells had a significantly greater disease burden (average 3.7 cm in the longest dimension; range 1.8–5.5 cm; $n = 6$), compared with those

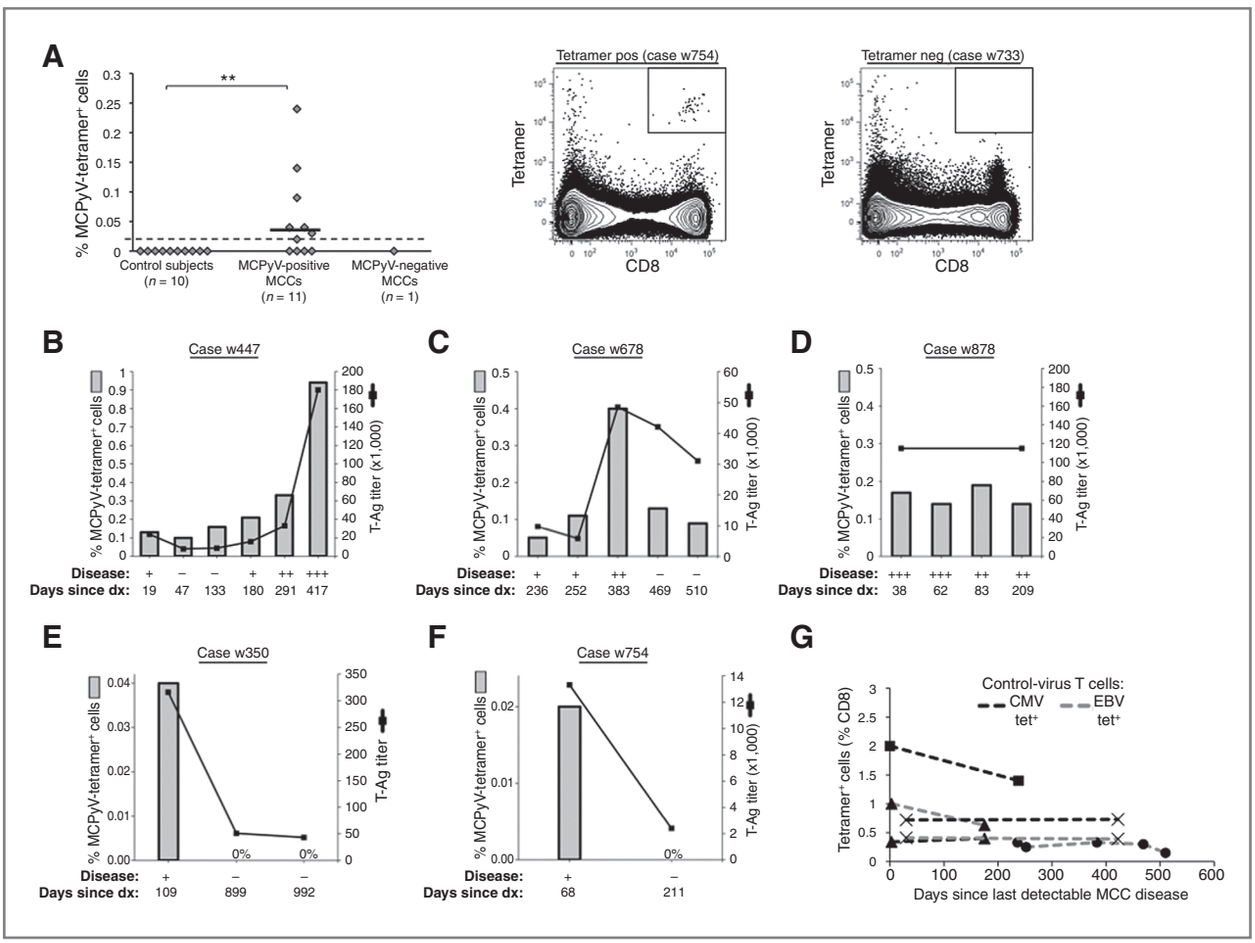

Figure 1. CD8 T cells specific for MCPyV were detected in the majority of patients with MCC and then tracked relative to tumor burden and anti–T-Ag antibodies. A, MCPyV-specific T-cell frequencies among patients or control subjects. Dashed line indicates the threshold of tetramer detection. Solid line indicates the median among patients with detectable MCPyV-specific T cells. All analyses were from the first available blood draw of subjects who were HLA-A*24 or HLA-A*23 positive. Representative flow cytometry plots are shown from cases that were tetramer-positive or tetramer-negative. **, $P < 0.01$, Fisher exact test. B–F, MCPyV-specific T cells (gray bars, percent of $CD3^+CD8^+$ cells) and anti-T-Ag antibody titers (black line) measured in serial blood draws from four patients with MCC at indicated times in their disease course. Days since diagnosis (dx) of primary tumor are indicated. Clinical extent of disease at time of blood draw is as indicated: (−) = none through (+++) = heavy burden. G, CMV-specific (black dashed line) or EBV-specific (gray dashed line) T cells were measured in serial blood draws from patients with MCC (circle, w678; X, w334; triangle, w672; square, w131) at the indicated times.

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without detectable T cells (average 0.7 cm; range 0.3–1.0 cm; $n = 3$; $P < 0.05$). Furthermore, among the 7 patients with detectable virus-specific T cells, blood was drawn near the time of known disease (at an average of 32 days since last detectable disease) and before completion of treatment. In contrast, in the 4 patients with no virus-specific T cells, blood was drawn at an average of 281 days after diagnosis or after last recurrence. As expected, in an HLA-compatible patient whose MCC tumor did not have detectable MCPyV oncoprotein expression (as assessed by CM2B4 or Ab3 antibody immunohistochemistry, data not shown; refs. 8, 19), no tetramer-positive T cells were found in peripheral blood obtained at a time point at which the patient had a sizable tumor burden. Collectively, our results show that circulating MCPyV-specific T cells are more likely to be found among patients with MCC with larger MCPyV-associated tumors.

MCPyV oncoprotein-specific T cells fluctuate with tumor burden and antiviral antibodies

The greater likelihood of detecting MCPyV-specific T cells among patients with large MCC tumors and in the ones with blood drawn near the time of disease prompted us to investigate how the frequency of virus-specific T cells changed over time in individual patients. As a baseline comparison of T-cell responses with other prevalent human viruses, we tracked the frequency of CD8 T cells specific for CMV or EBV in patients with MCC (without known clinically active CMV or EBV infection). There were no appreciable differences in the frequency of T cells specific for CMV or EBV over time (Fig. 1). In contrast, MCPyV-specific T-cell frequencies varied dramatically over time, correlating directly with tumor burden (Fig. 1). Interestingly, frequencies of T cells specific for the viral T-Ag oncoprotein also correlated directly with T-Ag antibody titers that have previously been reported to reflect tumor burden (20). Thus, both crosssectional (Fig. 1A) and longitudinal (Fig. 1B–F) studies indicate that MCPyV-specific CD8 T-cell levels increase with larger tumor burden and fall, sometimes to undetectable levels, with smaller or absent tumor burden.

MCPyV-specific and MCC-infiltrating CD8 T cells coexpress high levels of immune checkpoint receptors PD-1 and Tim-3

To determine the functional status of MCC-targeting T cells, we used a multiparameter flow cytometry phenotyping panel to characterize TILs and circulating MCPyV-specific T cells in patients with MCC. Because culture can alter protein expression patterns, specimens were phenotyped directly *ex vivo* for markers associated with costimulation (CD28, CD137), activation (CD69, CD137), and T-cell inhibition (PD-1, Tim-3, CTLA-4; Fig. 2 and Supplementary Fig. S2). T cells specific for CMV or EBV were used as controls. Activation and costimulation markers, CD28, CD69, and CD137 (4-1BB), suggestive of appropriate antigen recognition, were expressed on significantly more MCPyV-specific T cells from blood and MCC-infiltrating lymphocytes compared with other viruses (Fig. 2B).

PD-1 was expressed on a significantly higher percentage of MCC TIL (mean = 71 ± 8%; $n = 7$) and circulating MCPyV-specific T cells (96 ± 4%, $n = 5$) compared with T cells specific for CMV and EBV (Fig. 2B). Tim-3 was also significantly more likely (>3-fold) to be expressed on TILs from MCCs (34 ± 17%, $n = 7$) and MCPyV-specific T cells from PBMC (46 ± 21%, $n = 5$) as compared with control virus-specific T cells (Fig. 2B). Surface expression of another inhibitory molecule, CTLA-4, was generally low among TIL and CD8 T cells specific for MCPyV, CMV, and EBV (Fig. 2B).

Because simultaneous upregulation of multiple inhibitory receptors has been shown to be associated with T-cell dysfunction in other cancers (21), we evaluated the fraction of T cells that coexpressed key inhibitory receptors among TIL and PBMC specific for EBV, CMV, or MCPyV in patients with MCC (Fig. 3). The combination of PD-1 and Tim-3 coexpression was present among MCC TIL and MCPyV-specific PBMC at significantly higher levels (at least 8-fold higher) than on T cells specific for EBV or CMV ($P < 0.05$; Fig. 3B). More than 90% of those Tim-3$^+$ cells coexpressed PD-1. Furthermore, Tim-3 expression was most often observed among TIL with high-positive PD-1 levels as compared with cells with intermediate-positive PD-1 levels (Fig. 3C). MCPyV-specific PD-1$^+$ cells had a significantly higher median fluorescence intensity (MFI; >4-fold) compared with the PD-1$^+$ T-cell subset specific for CMV or EBV (Fig. 4A). We did not observe any differences in the density of Tim-3 or CTLA-4 expression (data not shown). Longitudinal studies revealed high PD-1 expression by MCPyV-specific CD8 T cells throughout the disease course, whereas there was minimal fluctuation in T cells specific for CMV or EBV (Fig. 4B).

To test function, we assayed the IFN-γ response of MCC-infiltrating lymphocytes and MCPyV-specific PBMC. Because none of the available TILs were from HLA-A24–positive patients, we used phorbol myristate acetate (PMA) and ionomycin to stimulate the cells. Of the four tested TIL samples, two failed to produce IFN-γ when stimulated with PMA/Ionomycin directly *ex vivo*. This dysfunction was reversed after a period of cell division initiated by phytohemagglutinin followed by a 6-day culture with IL-2 and IL-15 (Supplementary Fig. S2). Virus-specific PBMC responses could be evaluated in only one patient, w678, because others either lacked sufficient PBMC for this study or had baseline experimental characteristics (e.g., inability to expand *in vitro*) that were not interpretable as outlined in Materials and Methods. In this patient, the baseline number of MCPyV-specific CD8 T cells that secreted IFN-γ was markedly lower than would be expected on the basis of the number of virus-specific cells that were plated (Fig. 5; 1×10^5 CD8 T cells were plated, 0.87% of which were MCPyV tetramer-positive cells; data not shown). In contrast, while a similar number of EBV-specific CD8 T cells were plated (0.74% of 1×10^5 CD8 T cells), these cells produced a more robust IFN-γ response to the cognate antigen (Fig. 5).

Because PD-1 and Tim-3 are targets of agents in clinical development and are potentially relevant to the MCC

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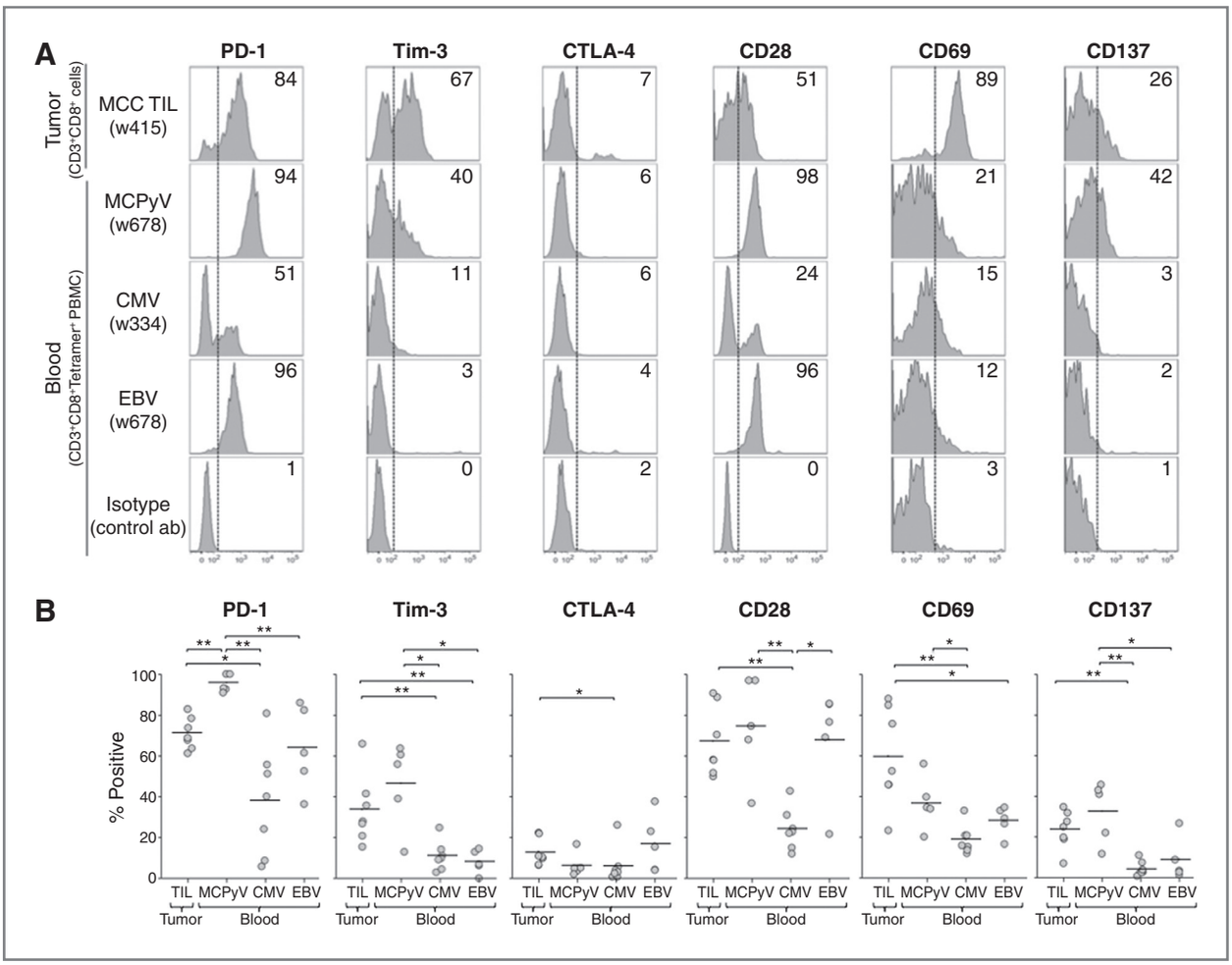

Figure 2. MCPyV-specific T cells and MCC TIL express multiple inhibitory receptors and activation markers. A, evaluation of three inhibitory receptors: PD-1, Tim-3, and CTLA-4, and three activation markers: CD28, CD69, and CD137 as assessed by flow cytometry. Representative histograms are gated on CD3+CD8+ TIL or CD3+CD8+Tetramer+ PBMC from blood. B, summary data for all the samples of patients with MCC analyzed: TILs from MCC tumors ($n = 7$); tetramer-positive PBMC specific for MCPyV ($n = 5$), CMV ($n = 7$), and EBV ($n = 5$). The horizontal line indicates the mean. Statistical comparisons were made between MCC TIL and MCPyV-specific PBMC and between all virus-specific T cells in the blood. *, $P < 0.05$; **, $P < 0.01$, Wilcoxon rank sum test.

immune response as described above, we tested whether blocking these inhibitory receptors could improve the function of MCPyV-specific T cells. CD8 T cells were exposed to cognate peptide and antibodies that functionally block PD-1, Tim-3 alone or in combination. After a short (20-hour) *ex vivo* stimulation, there was minimal peptide-specific IFN-γ response even in the presence of blocking antibodies (Fig. 5A). In contrast, when CD8 T cells were preincubated with the relevant peptide and blocking antibodies in a 7-day stimulation assay, we observed an augmented T-cell IFN-γ response to MCPyV peptide compared with similarly cultured cells to which blocking antibodies were not added (Fig. 5B). Although these results are encouraging, this study could only be carried out in a single patient because of experimental requirements including a high frequency of virus-specific T cells and a large starting blood volume.

In summary, we show that MCPyV-specific CD8 T cells from blood and MCC-infiltrating T cells predominantly coexpress PD-1 and Tim-3 inhibitory receptors that may prevent adequate control of MCC tumors *in vivo*. In addition, we show that MCPyV-specific CD8 T cells from peripheral blood secrete minimal IFN-γ in response to cognate peptide, and that this response can be augmented with antibodies targeting the relevant inhibitory receptors.

PD-L1 is expressed within MCC tumors and correlates with CD8 lymphocyte infiltration

Given the high level of PD-1 expression on MCC-infiltrating lymphocytes and MCPyV-specific CD8 T cells from blood, we investigated whether PD-1 ligand, PD-L1, was present within MCC tumors and whether it was associated with CD8 lymphocyte-infiltration. We evaluated PD-L1 and CD8 mRNA expression in 35 MCC tumors and protein expression in 13 formalin-fixed paraffin-embedded tumors. Expression of PD-L1 mRNA was correlated with CD8α

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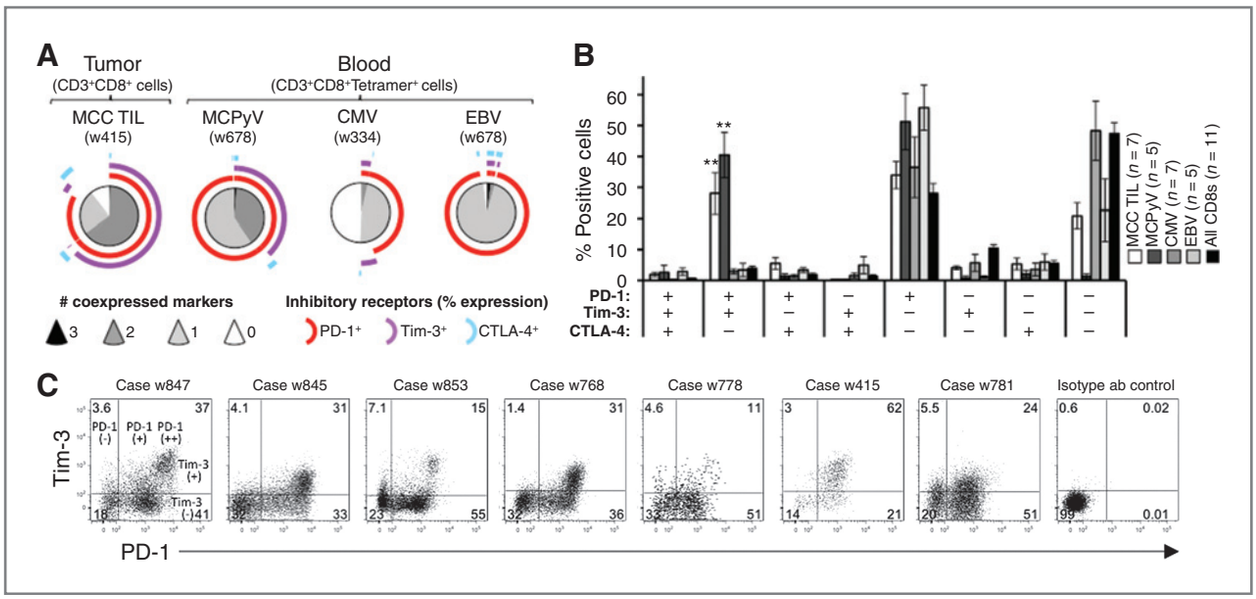

Figure 3. Coexpression of PD-1 and Tim-3 inhibitory receptors is elevated among MCPyV-specific T cells and MCC-infiltrating lymphocytes. A, coexpression of inhibitory receptors from four representative samples analyzed with SPICE software (35). Pie chart indicates number of coexpressed markers. Outer arcs correspond to the extent of indicated surface marker expression on $CD3^+CD8^+$ TIL or $CD3^+CD8^+Tetramer^+$ PBMC as assessed by flow cytometry. B, comparison of the fraction of cells that coexpress PD-1, Tim-3, and CTLA-4 in MCC CD8+ TIL ($n = 7$), PBMC specific for MCPyV ($n = 5$), CMV ($n = 7$), EBV ($n = 5$), and all $CD3^+CD8^+$ T cells ($n = 11$). The mean and SEM are shown. **, $P < 0.01$, Wilcoxon rank sum test. C, $CD3^+CD8^+$ TIL ($n = 7$) assessed for PD-1 and Tim-3 expression by flow cytometry. The appropriate isotype antibody controls are included in the rightmost panel. Three distinct populations of PD-1 expression are often detected. Relative expression is indicated on the first plot as (−) = negative, (+) = positive, (++) = high-positive.

mRNA ($R^2 = 0.6$; Fig. 6A). A nonoverlapping set (relative to the mRNA data) of archival tumor specimens was analyzed for PD-L1 and CD8 protein expression. Biopsy specimens from 9 of 13 patients (69%) had positive PD-L1 expression at levels that were weak ($n = 2$), moderate ($n = 4$), or high ($n = 3$) as assessed using a previously established scoring guide (17). Further analysis was carried out by grouping specimens as low-expressers (no or weak PD-L1 levels) and high-expressers (moderate or strong PD-L1 levels) as previously described (18). The intratumoral CD8 lymphocyte infiltrate was scored on a 0 to 5 scale (0 = absent to 5 = strong) as previously described (9). Consistent with the mRNA data, tumors with high PD-L1 expression were significantly more likely to have more intratumoral CD8 lymphocytes than those with low PD-L1 expression ($P < 0.05$; Fig. 6B). Representative histopathologic photographs are provided in Fig. 6C. This pattern of PD-L1 staining suggests that tumor infiltrating PD-1^+ T cells have a high

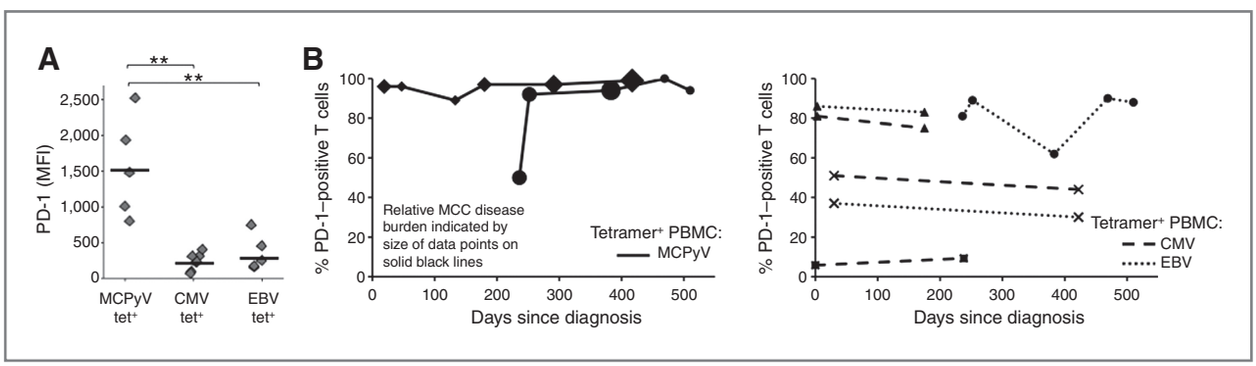

Figure 4. PD-1 is highly expressed on MCPyV-specific T cells and its expression is maintained throughout the MCC disease course. A, MFI of $CD3^+CD8^+$PD-1^+ T cells specific for MCPyV ($n = 5$), CMV ($n = 7$), and EBV ($n = 5$) measured in the first available blood draw from 12 patients with MCC. Most patients with MCC only had detectable tetramer-positive T cells for one of these viruses. Line indicates median. Tet^+ = tetramer-positive. *, $P < 0.05$; **$P < 0.01$, Wilcoxon rank sum test. B, percent PD-1 expression among $CD3^+CD8^+$ T cells specific for MCPyV (solid lines, left), CMV (dashed lines, right), or EBV (dotted lines, right) measured in serial blood draws from patients with MCC (diamond, w447; circle, w678; X, w334; triangle, w672; square, w131) at indicated times following diagnosis. Sizes of the black diamonds or circles on the solid black line represent relative disease burden among patients with MCC with MCPyV-specific T cells (smallest data points represent no detectable disease burden). Not all patients had T cells that were reactive to each tetramer, but all tetramer-positive T cells results are shown.

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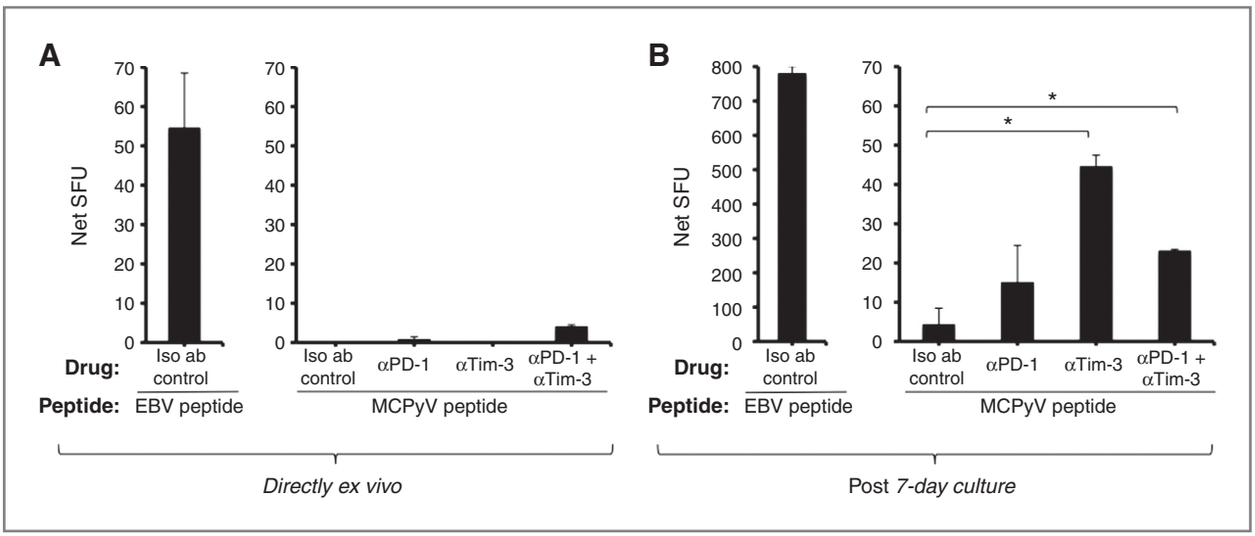

Figure 5. Culture with inhibitory receptor blocking agents augments MCPyV-specific T-cell function. A, PBMC from case w678 were analyzed directly *ex vivo* for ELISPOT-based IFN-γ cytokine production by CD8 T cells exposed to EBV or MCPyV peptide in the presence of indicated blockers or IgG isotype control antibody. Net SFU is the average SFU of indicated duplicate wells minus the average SFU in the negative control (media only) wells. Error bars represent mean ± SEM. B, PBMC from case w678 were cultured for 7 days as described in Methods and were assessed for IFN-γ production in an ELISPOT assay. A representative experiment is shown and analogous findings were obtained in a separate experiment. Error bars represent mean ± SEM. *, $P < 0.01$, Student *t* test.

chance of encountering their relevant inhibitory ligand in the MCC microenvironment.

Discussion

The purpose of this study was to investigate the mechanisms that prevent MCPyV-specific T cells from controlling MCC. Here, we show that MCPyV-specific T cells: (i) dynamically correlate in frequency with clinical disease burden and with antibodies against the viral oncoprotein (T-antigen), (ii) coexpress therapeutically-reversible markers of exhaustion, PD-1, and Tim-3 at far higher levels than T cells specific for other common human viruses, (iii) are likely to encounter the relevant inhibitory receptor ligand, PD-L1, within the MCC tumor microenvironment. These findings may help us optimize targeted approaches to overcome tumor immune evasion mechanisms in MCC.

While the concept that circulating antigen-specific CD8 T cells may fluctuate in number with viral (22) or tumor (18) load has precedent in the literature, to our knowledge, longitudinal tracking of tumor-specific T cells together with disease burden has not been previously reported. To track the frequency and function of Merkel polyomavirus-specific T-cell responses, we relied on an extensive collection of clinically annotated serial blood specimens from individual patients with MCC with variable disease burdens. In patients with MCC, we speculate that increased tumor burden (and the associated viral oncoprotein load) leads to the expansion of the oncoprotein-specific CD8 T-cell pool in the blood. An increase in MCPyV-specific CD8 T cells may thus provide a clinical biomarker of increasing disease. These data suggest that to obtain sufficient T cells for adoptive T-cell immunotherapy, it may be important to use PBMC acquired at times of higher tumor burden. In addition, because T-cell number increases with disease burden, there is a need for careful interpretation of immunotherapy efficacy data aimed at increasing tumor-specific T-cell frequency.

The presence and expansion of MCPyV-specific T cells with increasing tumor burden is highly suggestive that tumor immune escape mechanisms are active in MCC. T-cell dysfunction mediated by surface-expression of inhibitory molecules may, at least in part, explain why MCC tumors grow despite the presence of an immune response. We observed that among the majority of MCPyV-specific T cells in blood and MCC-infiltrating lymphocytes PD-1 and Tim-3 are simultaneously coexpressed, a combination that is often associated with chronic antigen exposure and reversible T-cell dysfunction (14, 21, 23–27). Our observations that most of the MCPyV-specific T cells in the blood are likely functionally exhausted, while at the same time, these cells increase in number in parallel with tumor burden, suggests that more than one population of MCPyV-specific cells is present. Memory T cells are generally segregated into effector-memory cells that traffic to sites of antigen and respond to peptide by secreting cytokines or executing a cytotoxic program, and central memory T cells that traffic to lymph nodes and are specialized for proliferation rather than effector functions (28). Our data suggest that the defect in MCC may preferentially involve the effector-memory population rather than the central-memory population, and this can be clarified in future work using markers for these cell subsets.

The present report suggests that the therapeutically targetable PD-1/PD-L1 pathway is particularly relevant in MCC. In contrast with prior studies that show upregulation

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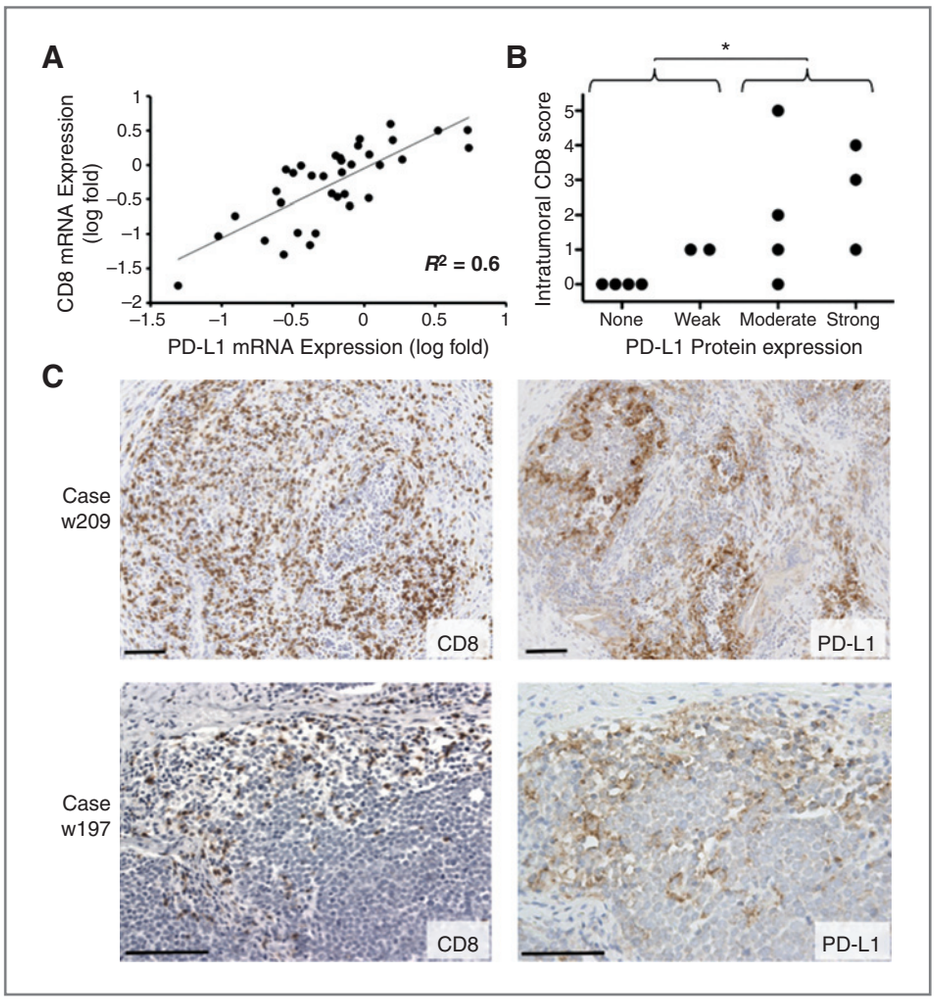

Figure 6. PD-L1 expression in MCC tumors correlates with CD8 lymphocyte infiltration. A, among 35 MCC tumors, CD8α and PD-L1 mRNA expression were closely correlated. B, correlation in an independent set of MCC tumors (compared with Figure 6A) between protein expression of PD-L1 and intratumoral CD8 infiltration in 13 MCC tumors. *, $P < 0.05$, Wilcoxon rank sum test. C, immunohistochemical analysis of CD8 infiltration (left) and PD-L1 expression (right) in two representative MCC tumors as assessed on serial sections of the same tumors. Scale bar: 100 μm.

of PD-1 with acute infection (22) or with increasing tumor stage (18), PD-1 expression on MCPyV-specific T cells was maintained at high levels throughout the MCC disease course. Furthermore, we observe a particularly high PD-1 receptor density level compared with control viruses, and speculate that this may be associated with decreased function. The relevant ligand, PD-L1, is often expressed within the tumor microenvironment (17, 29–31), and in melanoma, PD-L1–expressing tumor cells are often localized immediately next to TILs (32). In MCC tumors, using both histologic and mRNA-based analyses in independent cohorts, we observed that PD-L1 expression within the tumor microenvironment is positively correlated with the number of infiltrating CD8 lymphocytes. The heterogeneous expression of PD-L1 suggests that it is not necessarily confined to the tumor cells. Indeed, a recent study reports that in MCC tumors with PD-1–expressing T cells, PD-L1, and PD-L2 expression is mostly restricted to a subset of dendritic cells and macrophages (but not the cancer cells themselves; ref. 33). The presence of both PD-1 and PD-L1 within the tumor microenvironment suggests that the PD-1/PD-L1 inhibitory axis is a likely immune evasion strategy in MCC tumors. Importantly, the blockade of the PD-1/PD-L1 pathway has been recently shown to effectively induce durable tumor regression and stabilization of disease in a subset of patients with diverse types of cancer (12, 34).

There are several limitations to this study. We focused on a single, well-established MCPyV-specific epitope (11), which may provide a limited representation of the total antigen-specific immune response to MCC. We were limited in the number of longitudinal studies and antibody blockade experiments that were possible because of the rare aggressive nature of MCC, as well as the limited number of patients who have T cells that can be identified by the currently available peptide/HLA tetramer. The development of new peptide/HLA tetramers will expand the number of patients with MCC and the diversity of MCPyV-specific CD8 T cells that can be characterized.

In summary, this study shows that the frequency of MCPyV-specific CD8 T cells dynamically fluctuates with tumor burden and with viral oncoprotein-specific antibody titer. These cells are also characterized by high expression of multiple inhibitory and activation markers. Therefore, our data support the investigation of agents currently in clinical

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or preclinical trials, such as blockers of the PD-1/PD-L1 (12, 34) and of the Tim-3 axis (14, 15), or agonists of costimulatory molecules such as CD137 (13) in patients with advanced MCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: O.K. Afanasiev, N. Miller, K. Nagase, K. Paulson, J. Iyer, P. Nghiem
Development of methodology: O.K. Afanasiev, J. Iyer, P. Nghiem
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.K. Afanasiev, L. Yelistratova, K. Nagase, J. Iyer, D. Ibrani, P. Nghiem
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O.K. Afanasiev, L. Yelistratova, K. Nagase, D.M. Koelle, P. Nghiem
Writing, review, and/or revision of the manuscript: O.K. Afanasiev, L. Yelistratova, N. Miller, K. Nagase, K. Paulson, J. Iyer, D.M. Koelle, P. Nghiem
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O.K. Afanasiev, K. Nagase, K. Paulson, P. Nghiem
Study supervision: P. Nghiem

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Cancer Medicine

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ORIGINAL RESEARCH

Single-fraction radiation therapy in patients with metastatic Merkel cell carcinoma

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Abstract

Merkel cell carcinoma (MCC) is an aggressive, polyomavirus-associated cancer with limited therapeutic options for metastatic disease. Cytotoxic chemotherapy is associated with high response rates, but responses are seldom durable and toxicity is considerable. Here, we report our experience with palliative single-fraction radiotherapy (SFRT) in patients with metastatic MCC. We conducted retrospective analyses of safety and efficacy outcomes in patients that received SFRT (8 Gy) to MCC metastases between 2010 and 2013. Twenty-six patients were treated with SFRT to 93 MCC tumors located in diverse sites that included skin, lymph nodes, and visceral organs. Objective responses were observed in 94% of the measurable irradiated tumors (86/92). Complete responses were observed in 45% of tumors (including bulky tumors up to 16 cm). "In field" lesion control was durable with no progression in 77% (69/89) of treated tumors during median follow-up of 277 days among 16 living patients. Clinically significant toxicity was seen in only two patients who had transient side effects. An exploratory analysis suggested a higher rate of in-field progression in patients with an immunosuppressive comorbidity or prior recent chemotherapy versus those without (30% and 9%, respectively; $P = 0.03$). Use of SFRT in palliating MCC patients was associated with an excellent in field control rate and durable responses at treated sites, and with minimal toxicity. SFRT may represent a convenient and appealing alternative to systemic chemotherapy for palliation, for which most patients with oligometastatic MCC are eligible. SFRT may also synergize with emerging systemic immune stimulants by lowering tumor burden and enhancing presentation of viral/tumor antigens.

Introduction

Merkel cell carcinoma (MCC) is an aggressive skin cancer with a 46% disease-associated 5-year mortality [1]. Distant metastases are common (>30% of cases) and typically occur within 1–3 years following diagnosis [2]. As the median age of MCC patients is ~65 years, many patients are elderly with significant comorbidities. They are best managed by treatment that has minimal side effects, is convenient and cost-effective.

Traditional therapy for advanced metastatic MCC is cytotoxic chemotherapy or fractionated radiation. A small cell carcinoma chemotherapy regimen of carboplatin and etoposide is commonly used. Although most patients initially respond (reported response rate [RR] of 60%, 36% complete and 24% partial) [3], these responses are often not durable. Furthermore, chemotherapy is typically associated with significant side effects, and is limited to patients with good performance status. MCC is a radiosensitive cancer and fractionated radio-

therapy (FRT), typically delivered at 30 Gy over 10 fractions, is often effective for MCC metastases. However, FRT is logistically inconvenient, requiring multiple visits to an RT center.

Cellular immunity plays a particularly important role in MCC survival. Multiple forms of systemic immune suppression have been linked with an increased incidence of MCC [4]. Indeed, patients with systemic immune suppression have a significantly worse prognosis [5] independent of stage. Furthermore, the presence of intratumoral T-cell infiltration is associated in a stage-independent manner with improved MCC survival [6, 7]. Mouse model data suggest that single-fraction RT (SFRT) is more effective than FRT in augmenting local tumor immunity [8]. A likely contributor to this observation may be that cytotoxic CD8 T-cells that are stimulated and recruited to the tumor following SFRT are not killed by subsequent RT fractions. Although SFRT (8 Gy) has been used safely for decades for the treatment of bone metastases in other cancers [9–11], there are no reports of the use of SFRT for MCC. Furthermore, there are only very limited data regarding SFRT for nonbone metastases (NBM) in other cancer types.

There was a pressing clinical need for palliative therapy in patients who were not candidates for fractionated radiation therapy due to logistical issues and who had developed lesions that were chemotherapy-resistant and symptomatic. Given the known safety of SFRT (8 Gy) for bone metastases of many cancer types, we began to treat patients with this approach in 2010. Here, we report a retrospective analysis of our experience treating advanced MCC metastases with SFRT. The data suggest significant benefit, excellent tolerability, and a link to intact cellular immunity for this approach.

Patients and Methods

At initial evaluation, all patients were consented and enrolled into a FHCRC IRB-approved (#6585) prospective longitudinal database designed to assess outcomes relative to clinical features including stage and therapy.

Inclusion criteria for study cohort

All patients with metastatic MCC that received 8 Gy SFRT, with a minimum follow-up of 6 weeks between 1 December 2010 and 15 February 2013 were included in this retrospective study. The treatment was offered to all MCC patients who presented to our center with oligometastatic disease (typically 1–5 lesions) and who had not previously received RT to the target lesion(s). Patients with more than a single lesion were treated to some or all of their lesions, depending on disease burden, necessity for palliation of particular lesions, proximity to other major organs or neurovascular regions, and other patient considerations. Patients could receive other systemic therapies concurrent with and subsequent to SFRT without being censored from the study. There were no anatomic locations that were deemed inappropriate for SFRT. The dataset was finalized on 13 May 2013, after which no new data were included. Bony lesions could not be included in analyses of measurable disease response because they cannot be assessed for size/RECIST responses by computed tomography scans. Therefore, eight target tumors in five patients with bone metastases were assessed separately for symptom relief goals.

Treatment

SFRT (8 Gy) was delivered using electrons for skin and subcutaneous lesions, and photons using 3D conformal planning and IMRT (intensity modulated radiation treatment) for deeper tumors in the neck, mediastinum, and retroperitoneal regions. None of the patients were treated with stereotactic radiotherapy techniques. Standard doses of prophylactic antiemetic premedications including ondansetron and dexamethasone were routinely administered for 1–3 days beginning shortly before treatment of abdominal and retroperitoneal masses.

Monitoring/evaluation

All patients (except those with superficial lesions treated with electrons) had a CT scan for RT planning and responses tracked via CT scan. Superficial lesions were tracked by measurement with a ruler, and/or digital photography. Toxicity was graded using the Common Terminology Criteria of Adverse Events v3.0. RTOG/EORTC late radiation morbidity scoring schema. Of the 93 tumors treated using SFRT, efficacy analysis was carried out on 92 tumors (see Fig. 1). Response evaluation was by RECIST version 1.1 [12], modified only in that pretreatment lesion size reported was the longest dimension of all tumors including lymph nodes. When lesions were grouped in extreme proximity, they were irradiated with a single-targeted dose of 8 Gy and their sizes were measured as a single lesion. Complete palliation for bone metastasis was defined as complete resolution of pain, which was the only presenting symptom for these patients.

Data collection

For patients who received treatment at an outside facility following our initial assessment (of 93 tumors that received 8 Gy SFRT, 21 were treated at an outside facil-

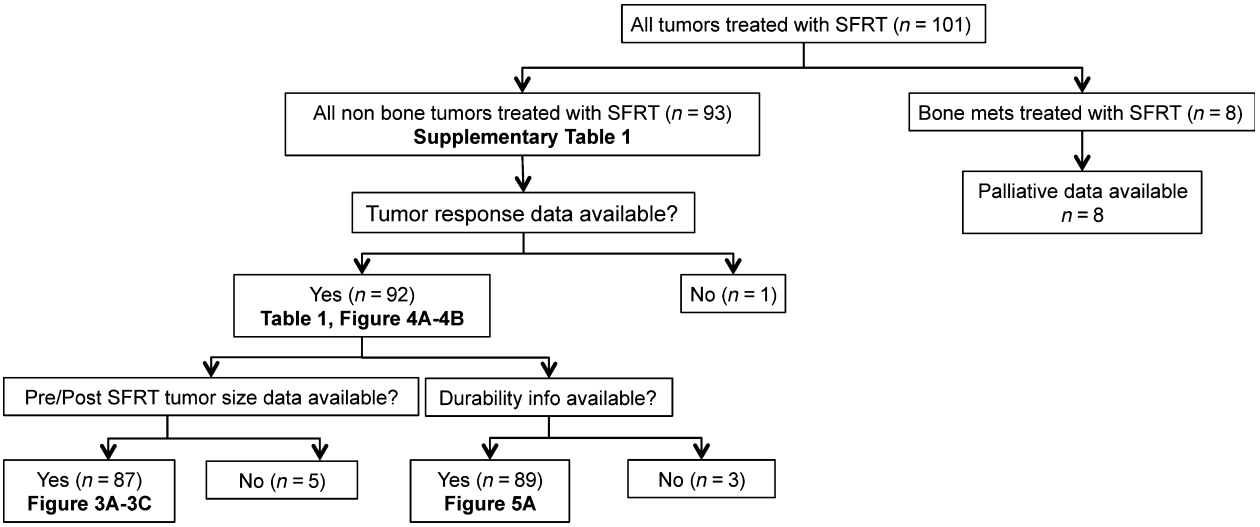

Figure 1. Flow diagram for 101 metastatic Merkel cell carcinoma lesions treated with single-fraction radiation therapy (SFRT). The diagram summarizes the available data used for the specified analyses and tables and figures in which those data are presented.

ity) all treatment records including physician notes, dosimetry records, and scan data were obtained and analyzed. Baseline patient and tumor characteristics including number, size, location of treated lesions, immune status, exposure to previous, and subsequent treatments, RT date, response to treatment, acute and late toxicity were recorded until last follow-up, death of the patient, or the data collection was terminated for this study on 13 May 2013.

Patient categories

Patients with chronic lymphocytic leukemia (CLL), HIV, those on immunosuppressive medications for solid organ transplant or autoimmune diseases, or exposed to cytotoxic chemotherapy for MCC prior to SFRT, were considered to have some degree of immunosuppression. We categorized patients into two categories, (1) low risk (LR) (patients with no known immune suppression or prior chemotherapy) and, (2) high risk (HR) (patients with known immune suppression or prior chemotherapy). Median time interval from chemotherapy to SFRT was 3.5 months (range 1.4–12.9 months).

Statistical analysis

Responses were noted on a per-tumor basis rather than a per-patient basis as some patients had multiple tumors that were treated on one or more dates. The RR was defined as the number of tumors (individual metastases) with complete (CR) or partial responses (PR) divided by the total number of evaluable tumors. However, analyses comparing LR to HR patient groups were conducted by considering multiple tumors in some patients by using generalized estimating equations (GEE). This method appropriately adjusts the variance of estimated effects in order to take into account the fact that some patients have multiple tumors. A log-link function was used to estimate the odds ratio of response between groups. Durability of response was calculated as the interval between SFRT and treated lesion progression, last follow-up date, or death (if treated lesion never progressed). Statistical analyses were carried out with SAS software (version 9.3; Cary, NC).

Survival curves

Survival that was free from progression of any treated lesion was estimated on a per-patient basis and was calculated as time between SFRT date and first progression of any treated lesion, death, or last follow-up (log-rank test was used to compare high- and low-risk patients). Durability of responses, tumor reduction percentage and magnitude of tumor responses were graphed using R statistical software, version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria) and ggplot2 (version 0.9.3.1, Hadley Wickham.).

Results

Patient and tumor characteristics

Between December 2010 and February 2013, 93 NBM in 26 patients were treated with SFRT. As shown in Table 1,

Table 1. Demographics of study cohort for RECIST-evaluable tumors.

Patient characteristics	*N*
Number of patients	26
Sex	
Male	22
Female	4
Median age at time of treatment (range)	68 years (54–96)
Number of MCC metastases treated with 8Gy SFRT evaluable by RECIST	92
Low-risk patients (no. tumors)	13 (32)
High-risk patients (no. tumors)	13 (60)
Median tumor size (range)	4 cm (1–19)
Characteristics of HR patients	No. patients (tumors)
Immunosuppressive illness (myelodysplasia) + medication (chronic methotrexate)	1 (3)
Medications (chronic methotrexate, anti-rejection medications)	2 (4)
Immunosuppressive illness (CLL or myelodysplasia) + prior chemotherapy	2 (6)
Only prior chemotherapy	8 (47)
Median interval between MCC diagnosis and SFRT (range)	568 (24–1987)
Low-risk patient tumors	669 (56–1987)
High-risk patient tumors	413 (24–429)
Median interval between first metastatic MCC lesion and SFRT (range)	207 (9–813)
Low-risk patient tumors	113 (9–445)
High-risk patient tumors	366 (35–813)
Patient outcomes	
Median follow up time from SFRT among all living patients (range)	277 days (104–699)
Low-risk patients	277 days (104–499)
High-risk patients	256 days (175–699)
Median time to treated lesion progression in days (no. of treated tumors that progressed)	
Low-risk tumors (3 of 32)	193 days
High-risk tumors (17 of 57) [1]	71 days

[1]Among 60 high-risk tumors, treated lesion progression data was available only for 57 tumors. For remaining 3 tumors treated lesion progression was unknown.

85% of patients in this study were male and 15% were female. The median age at time of treatment was 68 years (range 54–96 years). Thirteen of 26 patients (50%) were classified as high-risk patients (those with known immune suppression and/or prior chemotherapy; 60 tumors) whereas 13 were low-risk (no known immune suppression or prior chemotherapy; 33 tumors). Among high-risk patients, one had immune suppression alone (three tumors), two had both immune suppression and prior chemotherapy (six tumors), and 10 had prior chemotherapy (53 tumors). Median tumor size among all patients was 4 cm (range: 1–19 cm) and the average number of tumors treated per patient was 3.5 (range 1–28). The median interval between first metastatic MCC diagnosis and SFRT for LR tumors was shorter than for HR tumors, likely because the initial treatment for metastatic disease was chemotherapy for the HR tumors, meaning that SFRT started later.

Efficacy

A representative intensity modulated radiation therapy (IMRT) plan for targeting a mediastinal metastasis is shown in Figure 2. The posttreatment CT scan demonstrated complete resolution of the tumor, which was durable throughout the study period. Ninety-four percent of tumors responded (CR or PR) to SFRT. Five lesions were stable in size after SFRT, and one progressed (Fig. 3A). Although a higher fraction of treated tumors in LR patients had a CR (53%; 17 of 32) than of tumors in HR patients (37%; 22 of 60) this difference was not statistically significant ($P = 0.51$, GEE). As shown in Figure 3B, the size distribution for tumors that achieved CR or PR was similar. For lesions that could be assessed clinically (symptomatic and or superficial lesions) responses were typically noted by 7–10 days after therapy. For lesions requiring CT scan assessment, responses were usually seen at the first study following therapy (see Fig. 4). We did not observe spontaneous shrinkage of nontreated MCC tumors following SFRT (abscopal effect) in any of the cases during the study period. However, in the majority of patients we treated all the presenting lesions negating the ability to observe for potential abscopal effects.

Durability of responses

Eighty-nine tumors treated with SFRT also had data allowing assessment of durability beyond best response (Fig. 1). Sixty-nine of the 89 lesions (77%) did not progress during median follow-up of 8.4 months among living patients. CRs were durable, as none of the 40 tumors that achieved a CR recurred, regardless of the HR or LR status of the patient. Among the 20 lesions that progressed during the study period, the median time to treated lesion progression following SFRT was 2.5 months. Most strikingly, only 9% (three of 32) of tumors from patients in the LR group ever progressed

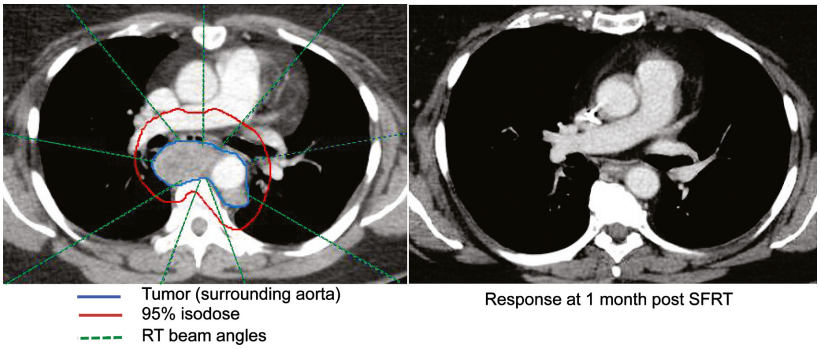

Figure 2. Radiotherapy plan and tumor response 1 month after SFRT. Left panel: A 56-year-old woman with recent stage IIIb MCC developed shortness of breath associated with a subcarinal paraesophageal lymph node metastasis (tumor outlined in blue, surrounding the aorta which is contrast-enhanced). She underwent SFRT, experienced no side effects from therapy, had full resolution of symptoms by day 5 after treatment, and by 1 month had a complete response as documented by CT scan (right panel). The red line represents the RT dose covering the tumor and the green dashed lines depict the nine RT beam angles directed at the tumor. The 95% isodose line in the radiotherapy plan closely conforms to the treated tumor in three dimensions, and dose was minimized to surrounding critical structures including spinal cord, heart and lungs. This tumor is included as lesion #23 in Table S1, and had not recurred as of the end of study period (11 months) or at last follow-up (22 months after SFRT). SFRT, single-fraction radiation therapy; MCC, Merkel cell carcinoma.

at the treated site, as compared with 30% (17 of 57) for tumors in patients in the HR group (odds ratio: 0.24, $P = 0.02$, 95% CI, 0.07–0.81, GEE; Fig. 5A). Furthermore, among tumors that ultimately progressed, the interval between SFRT and progression was longer for tumors arising in LR patients (Table 1; 193 days) as compared to high-risk patients (71 days).

Patient outcome

During the study period, two of 13 patients who were in the LR category died of MCC and one patient in this category died of an unknown cause, most likely not MCC (96-year-old man without evidence of MCC at time of death). In contrast, seven of 13 HR patients died of MCC during the study period. There were no deaths within 6 weeks of SFRT in either group. Median follow-up from first SFRT to last contact among the 16 surviving patients was 277 days (range, 104–699 days). Among the 10 patients who died, the time from SFRT to death ranged from 2.8 to 13.0 months with a median of 6.4 months. Survival free from progression of any treated lesion was significantly greater in LR patients than in HR patients ($P = 0.04$, log-rank test) and is plotted in Figure 5B.

Palliative efficacy for bone metastases

Patients had complete resolution of pain for 5/8 bone metastases (63%) treated with SFRT and the remaining three bone metastases had marked, but incomplete elimination of pain. All five complete palliation responses were durable throughout the study period.

Adverse events

No side effects of SFRT were noted in 24 of 26 patients, supporting a high degree of tolerability for the SFRT approach. The two patients who experienced side effects received therapy for large tumor volumes. Specifically, one patient underwent treatment of a 15 × 11 × 11 cm abdominal mass. He developed nausea and vomiting following SFRT that lasted 72 h and required hospitalization for IV hydration and antiemetic therapy. He had an excellent tumor response and did not require further treatment for over 10 months. Another patient who underwent simultaneous treatment of multiple subcutaneous, inflamed tumors developed a "flare pain" reaction that lasted <4 h. The patient presented to an emergency room and was successfully managed with nonsteroidal anti-inflammatory medication. There were no late/long-term effects attributable to SFRT.

Discussion

MCC is an aggressive, polyomavirus-associated skin cancer that is typically very radiosensitive. Development of metastatic MCC occurs in >30% of patients, however, options for treating metastatic disease are limited and unsatisfactory. In this retrospective study, we found a high RR (94%), excellent tolerability, and durable palliation for metastatic MCC lesions treated with SFRT. Indeed, objective responses were high among all MCC patients and durability of tumor responses was improved among patients without an immunosuppressive comorbidity or prior recent chemotherapy (low-risk patient group).

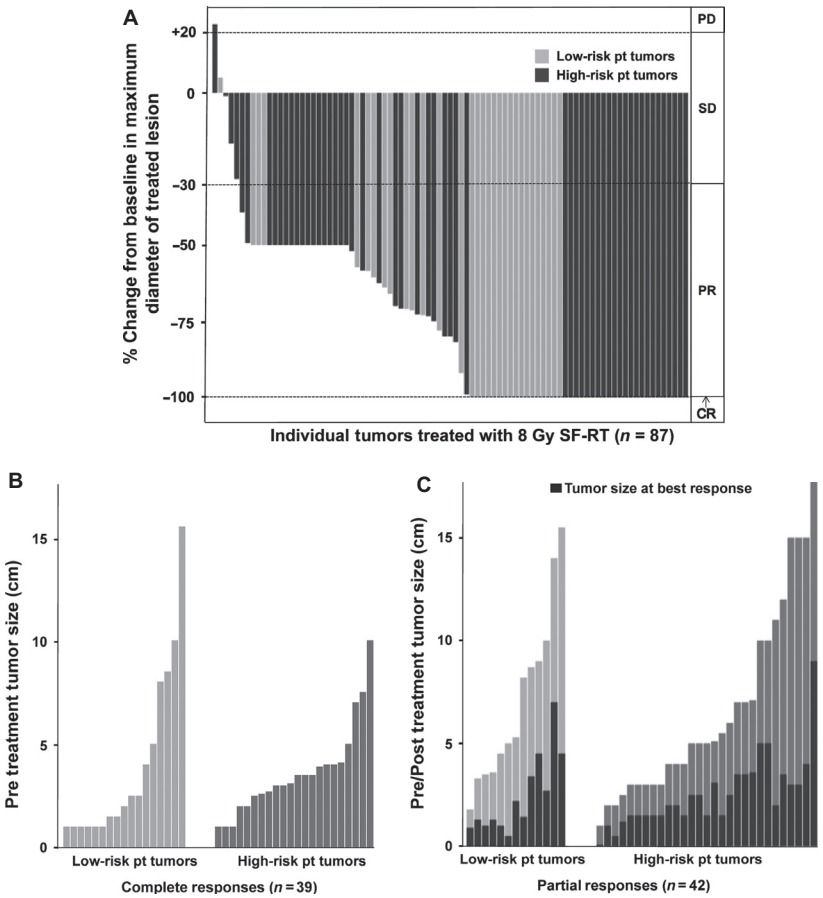

Figure 3. Tumor responses to SFRT: Of 92 tumors, 87 had both pre- and post-SFRT size measurements and could be included in this analysis (summarized in Fig. 1). In each panel, light gray bars represent low-risk patients who have no known immunosuppression and have not received prior chemotherapy; dark gray bars represent high-risk patients who have known systemic immune suppression and/or have received prior chemotherapy for MCC. (A) A waterfall plot of the percent change in largest treated lesion diameter at best response after SFRT as compared with baseline. Response criteria as per RECIST 1.1 [12] are as indicated on right of graph: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. (B) The pretreatment tumor size (largest dimension, in cm) for treated lesions that had a CR. 39 tumors with pretreatment measurements (22 high risk and 17 low risk) achieved CR. (C) The reduction in tumor size comparing pretreatment to best response for treated lesions that had a PR. Forty-two tumors (29 high risk and 13 low risk) achieved PR. The black bars in (C) (tumors with partial response) indicate tumor size at best response for each tumor. SFRT, single-fraction radiation therapy; MCC, Merkel cell carcinoma.

SFRT has been compared to fractionated RT for bone metastases in other cancers where it has been found to be safe and effective in the palliative setting [10, 11, 13, 14]. In a multicenter randomized study, Badzio et al. compared the efficacy of 4 Gy × 5 fractions with 8 Gy × 1 fraction for palliative therapy in bone metastases of breast, kidney, lung, prostate, and other cancers, and found that both treatments were equally effective [15]. Hoskin et al. [11] and Jeremic et al. [14] investigated the optimal SFRT dose by comparing results from 4, 6, and 8 Gy SFRT for bone metastasis from primary breast, prostate, thyroid, lung, kidney cancers, and myeloma. They found that the overall response rate in patients treated with 6 Gy (73%) and 8 Gy (78%) was significantly better than the response rate for patients treated with 4 Gy (59%), and that patients treated with 6 or 8 Gy achieved faster onset of pan relief than those that received 4 Gy. In our study, 94% of MCC tumors demonstrated a response to 8 Gy SFRT. This RR is higher than the 60–70% reported for bone metastasis [16]. However, this could be partially due to differences in the response evaluation for bone metastases versus the RECIST criteria used in our study. In addition, the higher RR could be reflective of the intrinsic radiosensitivity of MCC compared to other epithelial tumors (e.g., breast, lung, prostate, bowel, etc.) treated in the bone metastases studies.

While RRs of bone metastases to SFRT and FRT are comparable, data from randomized trials indicate responses are more durable following FRT [10]. For example, although a meta-analysis by Wu et al. [16]

reported similar RRs, retreatment was more frequent in patients that received SFRT (11–25%) as compared to FRT (0–12%) [16]. It is possible that the more durable palliative effect of FRT in bone metastases could be due to the significantly higher overall dose of 30 Gy in FRT, compared to 8 Gy in SFRT. In this study, we did not compare SFRT and FRT responses. Among patients who received SFRT, we found that responses (and symptom relief) were rapid among all patients but significantly more durable for the low-risk group than the high-risk group (Fig. 5A). There was no progression of tumors that achieved a CR in either patient group at the end of the study period (median follow-up of 7.6 months). It is plausible that in our study, the rapid initial responses typically seen in both high- and low-risk groups was due to the direct effect of RT on the tumor, independent of the immune response. In contrast, the improved durability of responses in the low-risk group may be due to the presence of a more functional immune system.

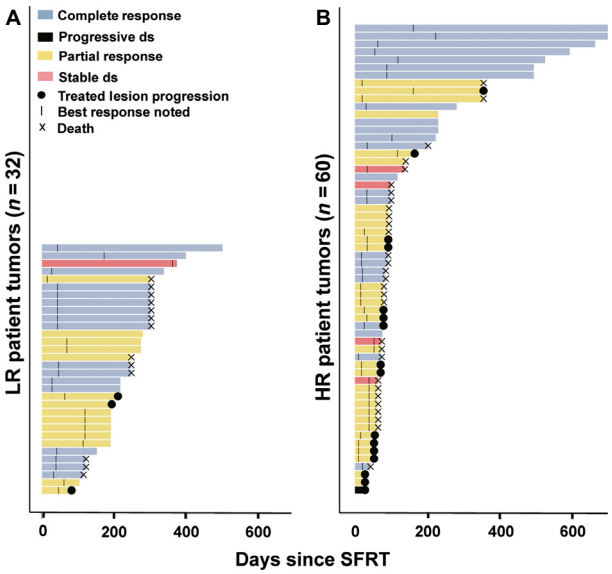

Figure 4. Durability of tumor responses. The period during which each treated tumor could be evaluated is plotted as a function of time in days since single-fraction radiation therapy (SFRT). "Events" were noted using symbols defined in the key at top left. Notably, none of the tumors that had a complete response (light blue bars) ever recurred. Tumors that have no symbol at the right side of their bar were not associated with progression or death at the time of last follow-up. (A) Represents tumors from low-risk patients that were treated with SFRT. (B) Represents tumors from high-risk patients treated with SFRT.

We hypothesized that SFRT might augment cellular immunity, a particularly important feature for control of MCC [6, 17]. There is substantial evidence that RT is capable of converting the irradiated tumor into an immunogenic hub. Animal studies suggest that low dose (2–4 Gy) SFRT can promote tumor immunity via major histocompatibility complex (MHC) up-regulation, antigen presentation, and vascular normalization [18]. At higher doses, SFRT likely retains these immunogenic effects, but also recruits T cells into the tumor and leads to greater direct tumor cell death due to apoptosis or necrosis [18]. Using a B16 mouse melanoma model, Lee et al. showed that SFRT (20 Gy) is more effective than fractionated radiation therapy (FRT; 45 Gy in 3 fractions) in controlling tumors though the total dose of radiation was far less

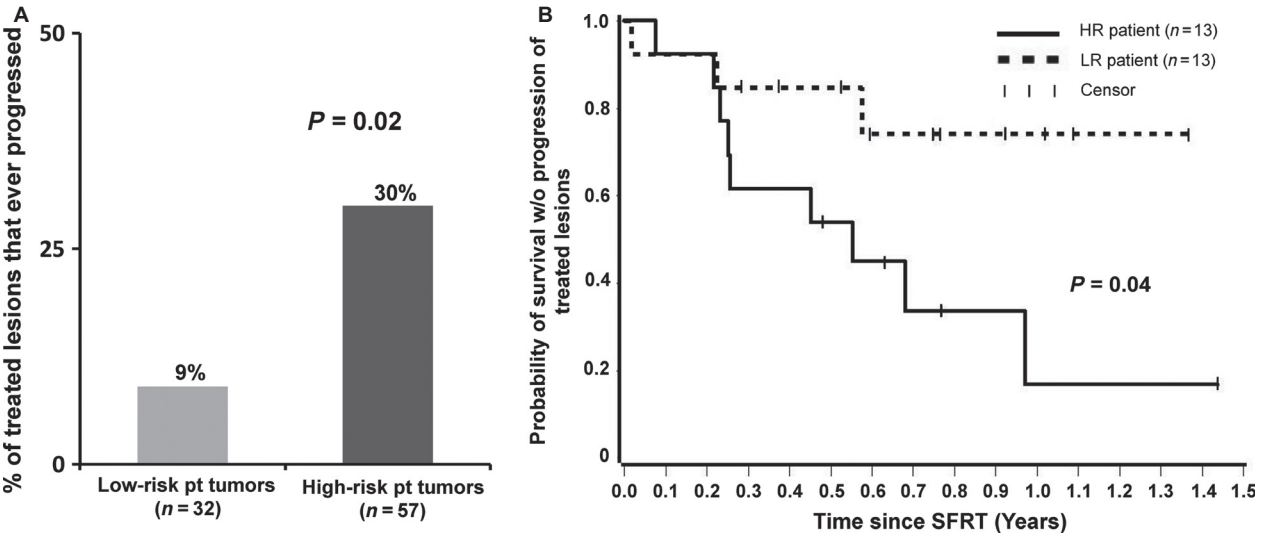

Figure 5. Risk of disease progression. (A) Risk of progression of single-fraction radiation therapy (SFRT)-treated lesions. 9% of tumors (three of 32) in low-risk patients progressed as compared to 30% of tumors (17 of 57) in high-risk patients ($P = 0.02$). (B) Survival without progression of treated lesions. The fraction of patients who were alive and remained free of progression from SFRT-treated lesion(s) is plotted as a function of years after SFRT.

[8]. In their model, the efficacy of SFRT was dependent on CD8 T-cells.

As an exploratory analysis, to determine whether a patient's immune status might have had bearing on the efficacy of SFRT, we segregated our cohort into two groups: low-risk (no apparent immune suppression or prior chemotherapy) and high-risk (known immune suppression and/or prior chemotherapy). Due to the size of our cohort (26 patients), the study lacked sufficient power to separately analyze patients who had prior chemotherapy as compared to those with other types of immune suppression. In addition, several patients had both risk factors. We thus combined patients with any form of immune suppression into one high-risk group. Indeed, several prior studies have demonstrated that chemotherapy can cause clinically significant and persistent T-cell immune suppression [19–21]. One study of 213 patients who received cytotoxic chemotherapy found that T-cell function was not normalized 12 months post chemotherapy [22]. In our cohort, the median time interval from chemotherapy to SFRT was 3.5 months (range 1.4 - 12.9 months), well within the documented interval for persistent T-cell suppression following chemotherapy. The patient receiving SFRT 12.9 months after chemotherapy also received SFRT at 9.4 and 10.4 months after chemotherapy and was thus classified as high risk. It is likely that other factors, besides immune function, could have contributed to the poorer outcomes in our high-risk patient cohort. For example, it is plausible that prior chemotherapy selected for radio-resistant tumor populations.

Although the vast majority of tumors in both groups responded, the durability of responses of treated lesions was significantly improved in low-risk patients (Fig. 5A). Although there are other possible explanations as noted above, the improved durability of responses in the low-risk group is analogous to the prolonged disease control seen with immune-based therapies for melanoma [23].

Although 94% of SFRT-treated tumors responded, we did not note spontaneous distant disease regression (abscopal effect) during the study period in any patient. There is evidence in a preclinical model that optimal dosing of radiation for inducing a systemic immune effect (compared to local effects studied by Lee et al.) may require more than a single fraction. Dewan et al. compared the efficacy of three RT regimens: 20 Gy × 1 fraction, 8 Gy × 3, and 6 Gy × 5 in combination with anti-CTLA4 (the latter had no effect on either model when given alone) and concluded that three or five radiation fractions provided a greater immune-stimulating effect at distant, nonradiated sites as compared to a single fraction of radiation [24].

There are several limitations of this study. Because this was a retrospective analysis, it is possible that inadvertent biases relating to patient selection, tumor response assessment or treatment techniques could have affected the results. In terms of patient selection, SFRT was offered to all patients with oligometastatic disease. The number of patients with immunosuppression not due to chemotherapy was limited and hence we were unable to separately analyze immune suppression in the absence of chemotherapy versus chemotherapy alone. There was variability in the timing of posttherapy evaluation of tumor responses. However, we do not believe that this factor would be likely to introduce systematic bias to the results. Regarding variability of treatment techniques, the majority (76%) of tumors were treated at our facility, minimizing interfacility variation. Moreover, patients treated elsewhere had results that were analogous to those from our own facility (94% response at our institution compared to 95% at outside institutions). Additionally, our cohort included patients with high disease burden (multiple or bulky tumors) in whom no single treatment modality was sufficient to control disease. Specifically, the majority (15/26) of patients treated with SFRT received one or more other systemic treatment modality concurrent with (four patients) or subsequent to (11 patients) SFRT that could have affected the efficacy of SFRT. These treatments included anti-CD137 antibody (three patients), pazopanib (six patients), somatostatin receptor analog (four patients), T-cell therapy (one patient), anti-PD1 (one patient), and cytotoxic chemotherapy (five patients). The median time between SFRT and initiation of subsequent systemic therapy in these 11 patients was 37 days. However, clinicians typically noted responses to SFRT within 7–10 days of treatment. In summary, because responses to SFRT were typically noted before initiation of systemic therapy, and because lesions in patients who received subsequent systemic therapy progressed at a similar rate (29%) to the entire group (23%), we believe that the efficacy observed at treated sites was likely due to SFRT rather than to other regimens.

This study demonstrates the safety and efficacy of SFRT for a wide range of metastatic MCC tumor locations. In a population with advanced age and comorbidities, the lack of toxicity and convenience of a single treatment approach is noteworthy. This study also demonstrates that MCC patients that have apparently normal immune status (low-risk) have significantly better response durability compared to those with known immune compromise and/or recent chemotherapy (high risk). This reinforces observations in MCC that strongly link immune status with disease control [6, 7]. In order to improve durability of response for the high-risk patients, it may be indicated to explore RT dose escalation and/or a modest increase in number of fractions. Further studies that correlate the immune status of patients with the immune milieu of the tumor microenvironment should be carried out to identify differences between

tumors that respond and those that do not. Such studies may also suggest strategies to augment antitumor immunity in unresponsive tumors. SFRT could be combined with emerging systemic immune stimulants such as immune checkpoint inhibitors to improve outcomes for this aggressive disease by lowering tumor burden and exposing viral/tumor antigens.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Tumor characteristics (^myelodysplasia, *MTX for rheumatoid arthritis, $^{+}$Kidney transplant).

ORIGINAL ARTICLE

PD-1 Blockade with Pembrolizumab in Advanced Merkel-Cell Carcinoma

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ABSTRACT

BACKGROUND

Merkel-cell carcinoma is an aggressive skin cancer that is linked to exposure to ultraviolet light and the Merkel-cell polyomavirus (MCPyV). Advanced Merkel-cell carcinoma often responds to chemotherapy, but responses are transient. Blocking the programmed death 1 (PD-1) immune inhibitory pathway is of interest, because these tumors often express PD-L1, and MCPyV-specific T cells express PD-1.

METHODS

In this multicenter, phase 2, noncontrolled study, we assigned adults with advanced Merkel-cell carcinoma who had received no previous systemic therapy to receive pembrolizumab (anti–PD-1) at a dose of 2 mg per kilogram of body weight every 3 weeks. The primary end point was the objective response rate according to Response Evaluation Criteria in Solid Tumors, version 1.1. Efficacy was correlated with tumor viral status, as assessed by serologic and immunohistochemical testing.

RESULTS

A total of 26 patients received at least one dose of pembrolizumab. The objective response rate among the 25 patients with at least one evaluation during treatment was 56% (95% confidence interval [CI], 35 to 76); 4 patients had a complete response, and 10 had a partial response. With a median follow-up of 33 weeks (range, 7 to 53), relapses occurred in 2 of the 14 patients who had had a response (14%). The response duration ranged from at least 2.2 months to at least 9.7 months. The rate of progression-free survival at 6 months was 67% (95% CI, 49 to 86). A total of 17 of the 26 patients (65%) had virus-positive tumors. The response rate was 62% among patients with MCPyV-positive tumors (10 of 16 patients) and 44% among those with virus-negative tumors (4 of 9 patients). Drug-related grade 3 or 4 adverse events occurred in 15% of the patients.

CONCLUSIONS

In this study, first-line therapy with pembrolizumab in patients with advanced Merkel-cell carcinoma was associated with an objective response rate of 56%. Responses were observed in patients with virus-positive tumors and those with virus-negative tumors. (Funded by the National Cancer Institute and Merck; ClinicalTrials.gov number, NCT02267603.)

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The programmed death 1 (PD-1) immune checkpoint pathway, which comprises the PD-1 T-cell coinhibitory receptor and its ligands PD-L1 and PD-L2 expressed on tumor and immune cells in the tumor microenvironment, mediates local immune resistance.[1] Monoclonal antibodies blocking this pathway are active against advanced tumors of several different types, providing a "common denominator" for cancer therapy.[2] PD-L1 expression in pretreatment tumor specimens may identify patients and tumor types that are more likely to have a response to PD-1 pathway blockade, and PD-L1 immunohistochemical tests were recently approved by the Food and Drug Administration to guide clinical decision making for patients with advanced non–small-cell lung cancer and melanoma who are candidates for anti–PD-1 therapy.[3] An elevated tumor mutational burden, creating new determinants (neoantigens) for immune recognition, has also been associated with tumor regressions in individual patients and the responsiveness of tumor subtypes to anti–PD-1 therapy.[4,5]

Merkel-cell carcinoma is a rare but aggressive skin cancer. For advanced Merkel-cell carcinoma, cytotoxic chemotherapy offers a median progression-free survival of only 3 months.[6,7] Merkel-cell carcinoma has long been considered to be an immunogenic cancer because it occurs more frequently and has a worse prognosis in immunosuppressed persons than in those with no immune suppression.[8] Two major causative factors have been identified: ultraviolet (UV) light and the Merkel-cell polyomavirus (MCPyV), whose large T antigen is expressed in tumor cells and inactivates p53 and Rb.[9] Approximately 80% of Merkel-cell carcinomas are associated with MCPyV, and patients with these carcinomas often produce MCPyV T-antigen–specific T cells and antibodies that increase with disease progression and decrease with effective therapy.[10-12] Virus-associated Merkel-cell carcinomas carry extremely low mutational burdens, in contrast to UV-induced, MCPyV-negative Merkel-cell carcinomas, which are characterized by a mutational load that is approximately 100 times as high.[13-15] Several studies have shown that approximately 50% of Merkel-cell carcinomas express PD-1 on tumor-infiltrating lymphocytes and express PD-L1 on tumor cells or infiltrating macrophages in an "adaptive resistance" pattern (with expression concentrated at the leading edges of the tumor), which suggests an endogenous tumor-reactive immune response that might be unleashed by anti–PD-1 or anti–PD-L1 drugs.[11,16-18]

The current study was undertaken to assess the efficacy of pembrolizumab, an anti–PD-1 therapy, in patients with advanced Merkel-cell carcinoma who had not previously received systemic therapy and to correlate treatment outcomes with tumor MCPyV and PD-L1 status.

Methods

Patients

Eligible patients were at least 18 years old and had distant metastatic or recurrent locoregional Merkel-cell carcinoma that was not amenable to definitive surgery or radiation therapy; measurable disease according to Response Evaluation Criteria in Solid Tumors, version 1.1; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 (on a scale of 0 to 5, with lower scores indicating less disability); and normal organ and bone marrow function.[19,20] Key exclusion criteria were previous systemic therapy for unresectable Merkel-cell carcinoma, a diagnosis of immunodeficiency or ongoing systemic immunosuppressive therapy, active autoimmune disease, concurrent second cancer, and active central nervous system metastases.

Study Design

This phase 2, single-group, Simon's two-stage, multicenter study was sponsored by the National Cancer Institute (NCI) and Merck and was developed by the authors in collaboration with the Cancer Immunotherapy Trials Network, the Cancer Therapy Evaluation Program, and Merck. According to Simon's two-stage design for efficacy estimation, at least one response among the first group of nine treated patients was required in order to enroll additional patients. Pembrolizumab, a humanized monoclonal IgG4 antibody (mAb) that blocks PD-1, was administered intravenously at a dose of 2 mg per kilogram of body weight every 3 weeks. Treatment was allowed to continue for a maximum of 2 years or until a complete response, dose-limiting toxic effects, or progressive disease occurred. Patients who appeared to have progression in target or non-

target lesions or to have new lesions were allowed to continue therapy if they were asymptomatic, had an ECOG performance status of 0 or 1, and had no evidence of rapid progression; patients were evaluated 4 weeks later to assess possible further progression.

The objective of this study was to determine the clinical efficacy of pembrolizumab as first systemic therapy for patients with advanced Merkel-cell carcinoma. The primary end point was the objective response rate measured according to RECIST, version 1.1.[19] Secondary end points were progression-free survival, overall survival, and duration of response. All adverse events were assessed according to NCI Common Terminology Criteria for Adverse Events, version 4.[21] Major exploratory objectives were to examine potential laboratory correlates for the clinical activity of pembrolizumab. The protocol is available with the full text of this article at NEJM.org.

STUDY OVERSIGHT

The protocol was approved by the institutional review board at each participating center, and the study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Good Clinical Practice guidelines. All the patients provided written informed consent before study entry. The principal investigators, in collaboration with the NCI and Merck, were responsible for the design and oversight of the study and the development of the protocol. The NCI was responsible for the collection and maintenance of the data. The manuscript was written and prepared by the authors with editorial oversight by the NCI. All the authors vouch for the accuracy and completeness of the data reported and adherence to the study protocol. No one who is not an author contributed to writing the manuscript.

CLINICAL ASSESSMENTS

All patients underwent computed tomographic scanning of the chest and abdomen (as well as other areas in which the target lesions occurred) at the time of screening and 12 weeks after starting therapy and at 9-week intervals thereafter. After 1 year of treatment, the scanning frequency was decreased to 12-week intervals. Evaluations of scans according to RECIST, version 1.1, were conducted at the institutional level, with central radiologic review performed by the NCI for patients who had a response. Pretreatment tumor specimens were obtained from all patients. The period between the pretreatment tumor biopsy and treatment initiation ranged from 7 days to 8.4 years (median, 5.2 months). Blood samples were drawn for correlative laboratory analyses at the time of radiologic studies. Post-treatment biopsies were obtained when clinically feasible.

TUMOR MCPYV STATUS

Recent or archival tumor specimens from all patients were assessed for expression of the MCPyV large T antigen oncoprotein through immunohistochemical analysis with a murine monoclonal IgG2b antibody (clone CM2B4, Santa Cruz Biotechnology).[22,23] Patients were also assessed for the presence of serum antibodies or circulating T cells specific for MCPyV oncoproteins (see the Methods section in the Supplementary Appendix, available at NEJM.org).[12] Because B-cell and T-cell reactivities against MCPyV oncoproteins are restricted to patients with MCPyV-positive tumors, patients with indeterminate tumor immunohistochemical results who were positive for serum antibodies or circulating MCPyV-specific T cells were categorized as having MCPyV-positive tumors.[11,12,24]

IMMUNOHISTOCHEMICAL TUMOR ANALYSIS

PD-L1 and PD-1 staining was performed at Merck Research Laboratories on formalin-fixed, paraffin-embedded tissue sections. Slides were subjected to heat-induced epitope retrieval and blocking of endogenous peroxidase before incubation with the primary antibody (anti–PD-L1 mAb clone 22C3 [Merck Research Laboratories] or goat anti–PD-1 polyclonal antibody [R&D Systems]). Antigen–antibody binding was visualized with the use of 3,3′-diaminobenzidine (Dako) for PD-L1 or Alexa Fluor 488 (Invitrogen) for PD-1. Samples were considered to be positive for PD-L1 if 1% or more of tumor cells expressed PD-L1. Tumor sections were also stained with anti-CD8 (clone 144B, Dako) to detect CD8+ T cells. Intratumoral CD8+ T cells (completely surrounded by tumor and not abutting stroma) were scored by a dermatopathologist who was unaware of patient characteristics, as described previously[25] and in Figure S2 in the Supplementary Appendix.

Selected specimens were assessed with multispectral immunohistochemical analysis, which provided simultaneous detection and quantitation of neuron-specific enolase (Merkel tumor cells), CD8, CD68 (macrophages), PD-1, and PD-L1 (see the Methods section in the Supplementary Appendix).

STATISTICAL ANALYSIS

Patients who received at least one dose of pembrolizumab were included in the safety and efficacy analyses. Data are reported as of February 12, 2016. Radiologic and physical-examination assessments according to RECIST, version 1.1, were used to determine treatment responses.[19] The best overall response was defined as the best response recorded from the start of the treatment until disease progression or recurrence. The objective response rate was calculated as the percentage of patients who had a complete or partial response that was confirmed by a subsequent radiologic imaging study according to RECIST, version 1.1,[19] among all the patients who received at least one dose of pembrolizumab and had at least one evaluation during treatment. Clopper–Pearson exact confidence intervals were generated for the response rate. Time to response was defined as the time interval between the first administered dose of the drug and the date of first response. Duration of response was defined as the time interval between the date of first response and the date of disease progression or death. For patients who did not have disease progression or die, the end date for response duration was the later of the last disease assessment or last treatment administration. Progression-free survival was defined as the time interval from the date of the first dose of pembrolizumab to the date of disease progression or death, whichever occurred earlier, and was estimated with the use of the Kaplan–Meier method.[26] An unconditional exact test was used to assess associations between PD-L1 expression and clinical response or viral status.[27] The Mann–Whitney U test was used to compare distributions of CD8 scores between virus-positive patients and virus-negative patients.

Table 1. Patient Characteristics.*

Characteristic	All Patients (N=26)	Patients with Virus-Positive Tumors (N=17)	Patients with Virus-Negative Tumors (N=9)
Age at enrollment — yr			
Mean	70.5±8.1	67.5±6.0	76.3±8.6
Median (range)	68 (57 to 91)	67 (57 to 83)	76 (64 to 91)
Sex — no. (%)			
Female	10 (38)	4 (24)	6 (67)
Male	16 (62)	13 (76)	3 (33)
Disease stage at study entry — no. (%)			
IIIB	2 (8)	2 (12)	0
IV	24 (92)	15 (88)	9 (100)
Previous duration of disease — wk†			
Mean	58.8±56.8	71.3±63.5	35.2±32.6
Median (range)	39 (3 to 227)	53 (3 to 227)	27 (5 to 104)
Baseline extent of disease — mm‡			
Mean	81.7±53.9	88.7±63.1	68.6±28.7
Median (range)	69 (13 to 182)	62 (13 to 182)	75 (36 to 123)

* Plus–minus values are means ±SD.
† Previous duration of disease was measured from the date of diagnosis to the date of the first dose of study treatment. An unknown day of diagnosis was imputed as mid-month for one patient.
‡ The extent of disease was measured before treatment initiation as the sum of the longest diameters of tumor target lesions.

RESULTS

PATIENT CHARACTERISTICS

A total of 26 patients with stage IIIB or IV Merkel-cell carcinoma and an ECOG performance status of 0 or 1 were enrolled from January 2015 until December 2015 and received at least one dose of pembrolizumab. Patient characteristics at baseline are shown in Table 1. The median age of the patients was 68 years (range, 57 to 91). Nine patients were classified as having MCPyV-negative tumors (35%), and 17 were classified as having MCPyV-positive tumors (65%). No patients had received previous systemic therapy for advanced Merkel-cell carcinoma; however, one patient had received adjuvant chemotherapy more than 6 months before beginning study treatment.

CLINICAL ACTIVITY

The characteristics of response to anti–PD-1 are shown in Figure 1. A total of 25 patients had at least one tumor assessment during treatment, of whom 14 had a confirmed response (4 with a complete response and 10 with a partial response), representing an objective response rate

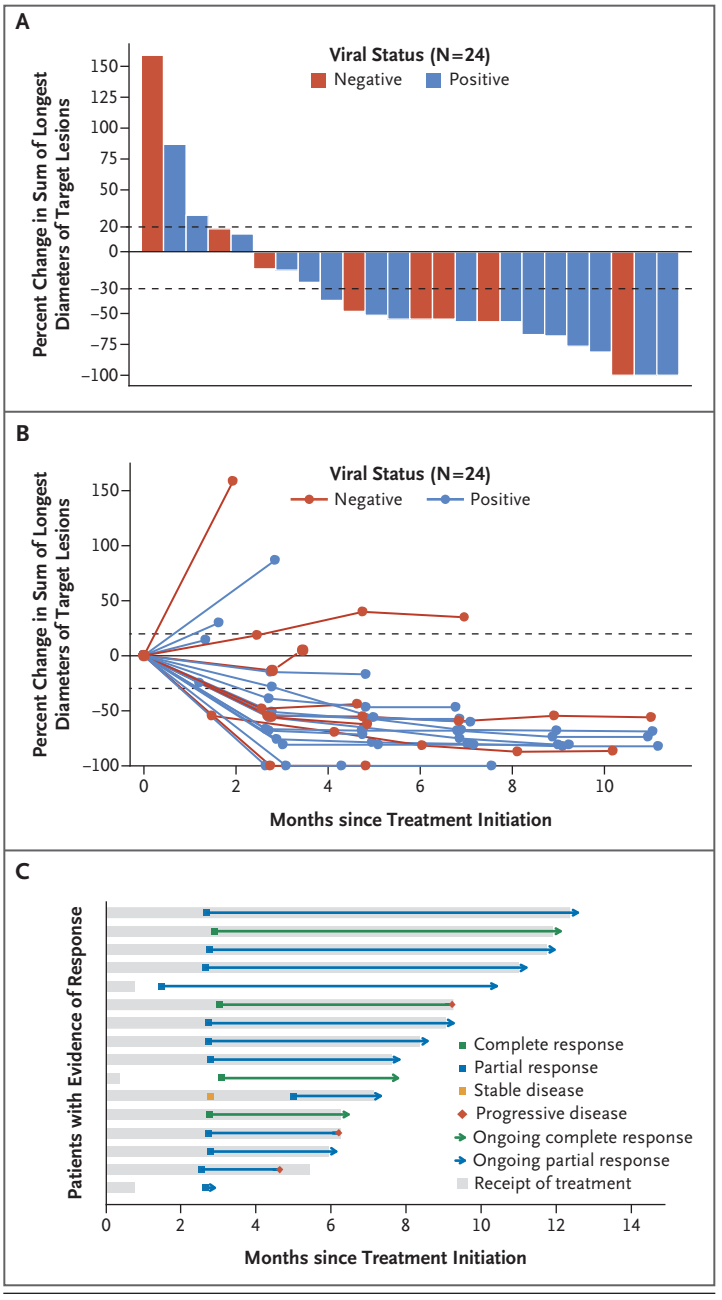


Figure 1. Clinical Characteristics of Tumor Response to Pembrolizumab in Patients with Merkel-Cell Carcinoma.

Panel A shows the maximum percent change from baseline in the sum of the longest diameters of target lesions in the 24 patients who underwent radiologic evaluation after treatment initiation. Viral status refers to whether patients had tumors that were positive or negative for the Merkel-cell polyomavirus (MCPyV). Horizontal dashed lines indicate criteria in the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, for partial response (≥30% decrease in the sum of the longest diameters of target lesions, with the assumption of no new lesions) and progressive disease (≥20% increase in target-lesion diameters). Data from 2 of the 26 patients are not shown: one had radiographic evidence of disease progression with new lesions, without radiographic evaluation of target lesions, and thus discontinued therapy; the other had not yet undergone radiologic evaluation after initiating pembrolizumab therapy, as of the date of analysis. Panel B shows the kinetics of change in target-lesion diameters over time during pembrolizumab therapy. Rapid and durable reductions in target-lesion diameters were observed in most patients. Two patients with confirmed responses (1 with a partial response and 1 with a complete response) subsequently had progression in nontarget lesions, which are not represented here. Panel C shows the characteristics of 16 patients who initially had evidence of response according to RECIST, version 1.1. Each horizontal bar represents 1 patient. Most responses were observed at the first tumor assessment (approximately 12 weeks after treatment initiation), and 13 of the 16 initial responses (81%) were ongoing at the time of analysis. A total of 14 patients had a confirmed response (2 of whom later had progressive disease); in addition, 1 patient with an unconfirmed partial response continues to receive therapy (bottom bar), and one patient with a transient partial response (i.e., not confirmed by follow-up computed tomographic scanning) subsequently had progressive disease (next-to-bottom bar).

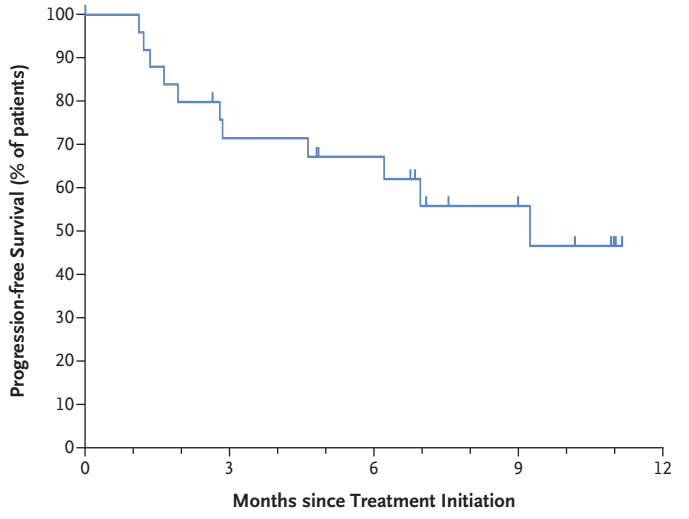


Figure 2. Kaplan–Meier Curve Showing Progression-free Survival among 26 Patients with Merkel-Cell Carcinoma Who Received Pembrolizumab.

Progression-free survival was measured from treatment initiation to disease progression or death, whichever occurred first. Data from patients without an event were censored at the last date of disease assessment (tick marks). The estimated rate of progression-free survival at 6 months was 67% (95% confidence interval [CI], 49 to 86). The median progression-free survival was 9 months (95% CI, 5 months to not reached). As of February 12, 2016, a total of 11 events of disease progression or death had occurred.

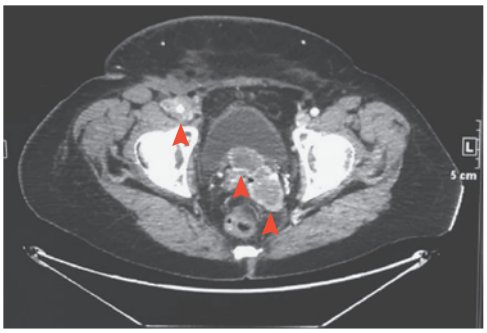
Figure 3 (facing page). Response to Pembrolizumab in a Patient with Stage IV Merkel-Cell Carcinoma.

This 69-year-old woman received a diagnosis of a primary cutaneous lesion on the right knee and was treated with wide local excision, sentinel lymph-node biopsy, and inguinal lymph-node dissection in November 2013. Recurrent Merkel-cell carcinoma developed in September 2014, with a pelvic mass measuring 11 cm by 7 cm by 14 cm, which was associated with worsening lymphedema and moderate-to-severe right hydroureteronephrosis requiring a ureteral stent. The patient received radiation therapy to the pelvic mass but in January 2015 was found to have new peritoneal and lymph-node metastases (Panel A, red arrows), as well as several subcutaneous metastases on the right thigh and just below the site of excision of the primary tumor (Panel B; red arrow indicates the site of previous excision of the primary tumor, just below the knee). As shown, these metastatic sites regressed rapidly during anti–programmed death 1 (PD-1) therapy. Also shown are the results of pathological analysis of the primary tumor (Panel C, left) and adjacent post-treatment subcutaneous metastasis (Panel C, right) with multispectral immunohistochemical analysis. Orange indicates Merkel carcinoma cells expressing neuron-specific enolase, yellow CD8+ T cells, red CD68+ macrophages, white PD-1, green the PD-1 ligand PD-L1, and blue nuclear DNA stained with 4′,6-diamidino-2-phenylindole (DAPI). Analysis of the archival biopsy specimen shows an immune infiltrate that is most intense at the tumor–stromal interface, including CD68+ macrophages and CD8+ T cells infiltrating the tumor parenchyma. PD-1 is expressed on 56% of CD8 cells in this microscopic field. PD-L1 is expressed on tumor cells (10% of tumor cells in this field, blue arrows) and macrophages (43% of macrophages in this field, red arrows) and is seen immediately adjacent to PD-1+ lymphocytes. Analysis of the post-treatment biopsy specimen shows a diffuse immune-phagocytic infiltrate and no evidence of residual tumor. The immune infiltrate includes CD68+ macrophages and CD8+ T cells, with an early lymphoid aggregate (white star) where PD-1 and PD-L1 expression is observed.

of 56% (95% confidence interval [CI], 35 to 76). In addition, 1 patient with an unconfirmed partial response continues to receive treatment. One of the 25 patients (4%) had stable disease, and 9 (36%) had progressive disease. The 26th patient has not yet undergone a radiologic assessment for response. Twelve of the 14 confirmed responses (86%) were ongoing at last follow-up. The median follow-up was 33 weeks (range, 7 to 53). With respect to tumor viral status among the 25 patients whose response to treatment could be evaluated, 10 of 16 patients (62%) with virus-positive tumors and 4 of 9 (44%) with virus-negative tumors had an objective response (Fig. 1A and 1B). Among all 26 patients, the median treatment duration was 27 weeks (range, 3 to 57), and 14 patients continue to receive treatment. Among 14 patients with an objective response, the response duration ranged from at least 2.2 months to at least 9.7 months (Fig. 1C). Kaplan–Meier analysis yielded an estimated rate of progression-free survival at 6 months of 67% (95% CI, 49 to 86) (Fig. 2). Among 9 patients who had progressive disease, progression occurred in preexisting target lesions (4 patients), new metastatic sites (2 patients), or both (3 patients). In 2 of the 14 patients with confirmed responses (14%), disease progression developed later, with new metastatic sites in the central nervous system (frontal lobe of the brain in one patient and leptomeningeal sites in the other patient).

Aspects of the clinical course in a virus-positive patient with a partial tumor regression are shown in Figure 3. This patient with multiorgan metastases showed a substantial reduction in pelvic tumors at the first radiologic evaluation (Fig. 3A) and a complete regression, as assessed by pathological evaluation, of a subcutaneous metastasis 3 weeks after initiating pembrolizu-

A



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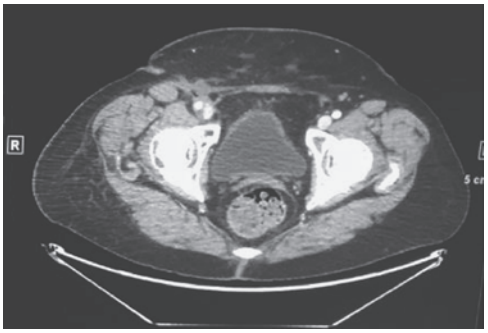

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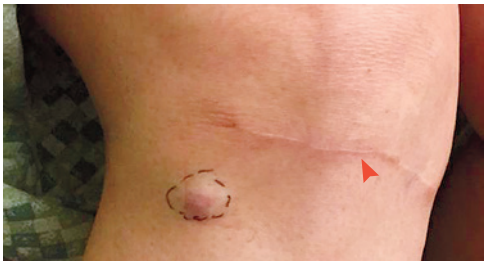

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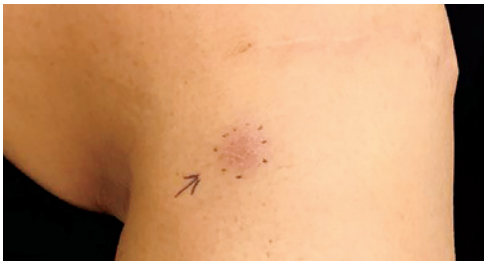

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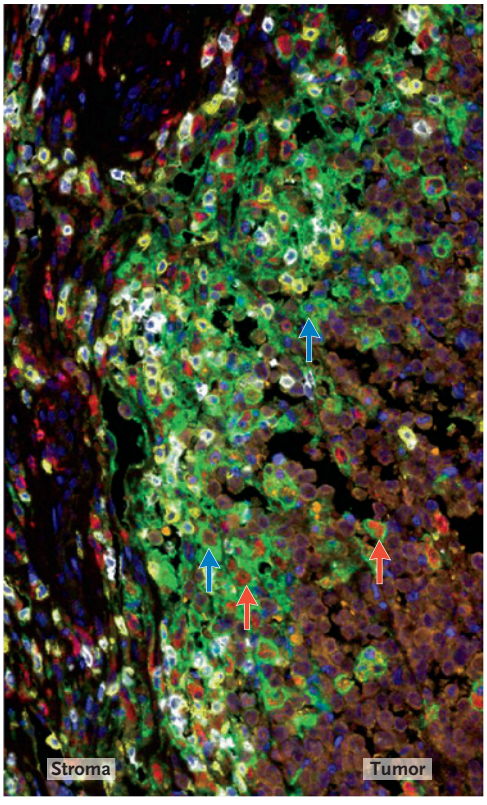
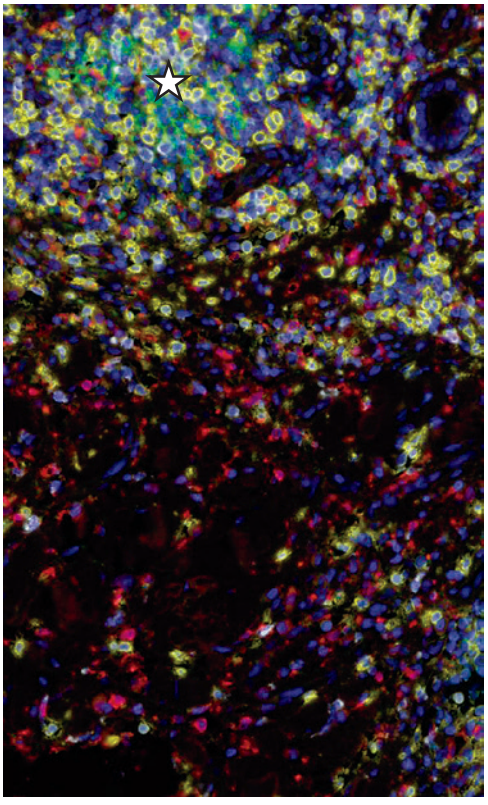


Archival Biopsy Specimen
of Primary Merkel-Cell Carcinoma



Post-treatment Biopsy Specimen
of Subcutaneous Metastasis

mab therapy (Fig. 3B, and Fig. S1 in the Supplementary Appendix). Multispectral immunohistochemical analysis of an archival specimen of the primary tumor showed PD-L1+ tumor cells and infiltrating macrophages abutting PD1-expressing CD8 cells. Examination of the post-treatment biopsy sample from the adjacent regressing subcutaneous metastasis (which was present at the time of the first pembrolizumab dose) showed inflammation, as evidenced by infiltrates of CD68+ macrophages and CD8+ T cells, without evidence of tumor (Fig. 3C, and Fig. S1 in the Supplementary Appendix).

SAFETY

Treatment-related adverse events of any grade occurred in 77% of the patients. The most common adverse events were fatigue and laboratory abnormalities (Table S1 in the Supplementary Appendix) — findings that were similar to those in previous reports.[3,28] Grade 3 or 4 treatment-related adverse events were observed in 4 of the 26 patients (15%). Two patients had a grade 4 adverse event; one had myocarditis after having received one dose of pembrolizumab and another had elevated levels of alanine aminotransferase and aspartate aminotransferase after having received two doses of pembrolizumab. Both patients had a reduction in the adverse events after discontinuation of pembrolizumab and initiation of glucocorticoid treatment. Both also had tumor regressions that are ongoing (one partial and one complete) (Fig. 1C).

CORRELATION OF TUMOR PATHOLOGIC FEATURES WITH CLINICAL OUTCOMES

PD-L1 expression could be evaluated in pretreatment tumor specimens from 25 of the 26 patients. PD-L1, the major ligand for PD-1, can be expressed on tumor cells or on infiltrating immune cells (such as macrophages), which are a prominent feature of Merkel-cell carcinoma (Fig. 3C).[17,18,29] Furthermore, PD-L1 expression can occur on either cell type in an "adaptive" pattern (i.e., at the interface with infiltrating lymphocytes and presumably promoted by inflammatory cytokines) (Fig. 4A) or on tumor cells in a "constitutive" pattern (i.e., uniform expression not associated with infiltrating lymphocytes, probably driven by genetic or epigenetic events intrinsic to tumor cells) (Fig. 4B). In our analysis of tumors from 25 patients, neither PD-L1 expression on tumor cells (Fig. 4C) nor expression on infiltrating immune cells (not shown) correlated significantly with clinical response to pembrolizumab. PD-L1 expression was more frequent in virus-positive tumors than in virus-negative tumors (71% vs. 25%, $P=0.049$) (Fig. 4D). There was no significant correlation of intratumoral CD8 T-cell infiltration with clinical response or with viral status (Fig. S2 in the Supplementary Appendix).

DISCUSSION

The PD-1–blocking antibodies pembrolizumab and nivolumab are promising therapies for patients with advanced metastatic melanoma and non–small-cell lung cancer, and nivolumab was approved for the treatment of renal-cell carcinoma, on the basis of clinical trials showing durable antitumor efficacy and a favorable safety profile.[3,28,30-33] Clinical trials in additional cancer types have shown encouraging results,[5,34-36] whereas some other cancers appear to be refractory to anti–PD-1 therapy.[3] Potential factors associated with response, including tumor PD-L1 expression, the presence of CD8 T cells at the "invading tumor margin,"[37] and high tumor mutational load, are currently under investigation.

Merkel-cell carcinoma exemplifies the intersection of several exploratory biomarker categories: it is often associated with PD-L1 expression and CD8 infiltrates, and it can have a high mutational burden (carcinogen [ultraviolet light]–induced) or can be virus-associated. The presence of oncogenic viruses in virus-associated cancers, wherein viral antigens serve as tumor-specific antigens, has recently been proposed as a potential mechanistic marker that can predict response to anti–PD-1 therapy. More than 20% of all cancers worldwide are virus-associated and may have low or modest mutational burdens owing to tumorigenesis driven by the dominant effects of viral oncogenes. Viral antigens are foreign and thus potentially strong immune stimulants, and many virus-associated tumors are characterized by robust immune infiltrates and PD-L1 expression.[17,38]

These observations provide a strong rationale for assessing the efficacy of PD-1 pathway blockade in patients with advanced, previously un-

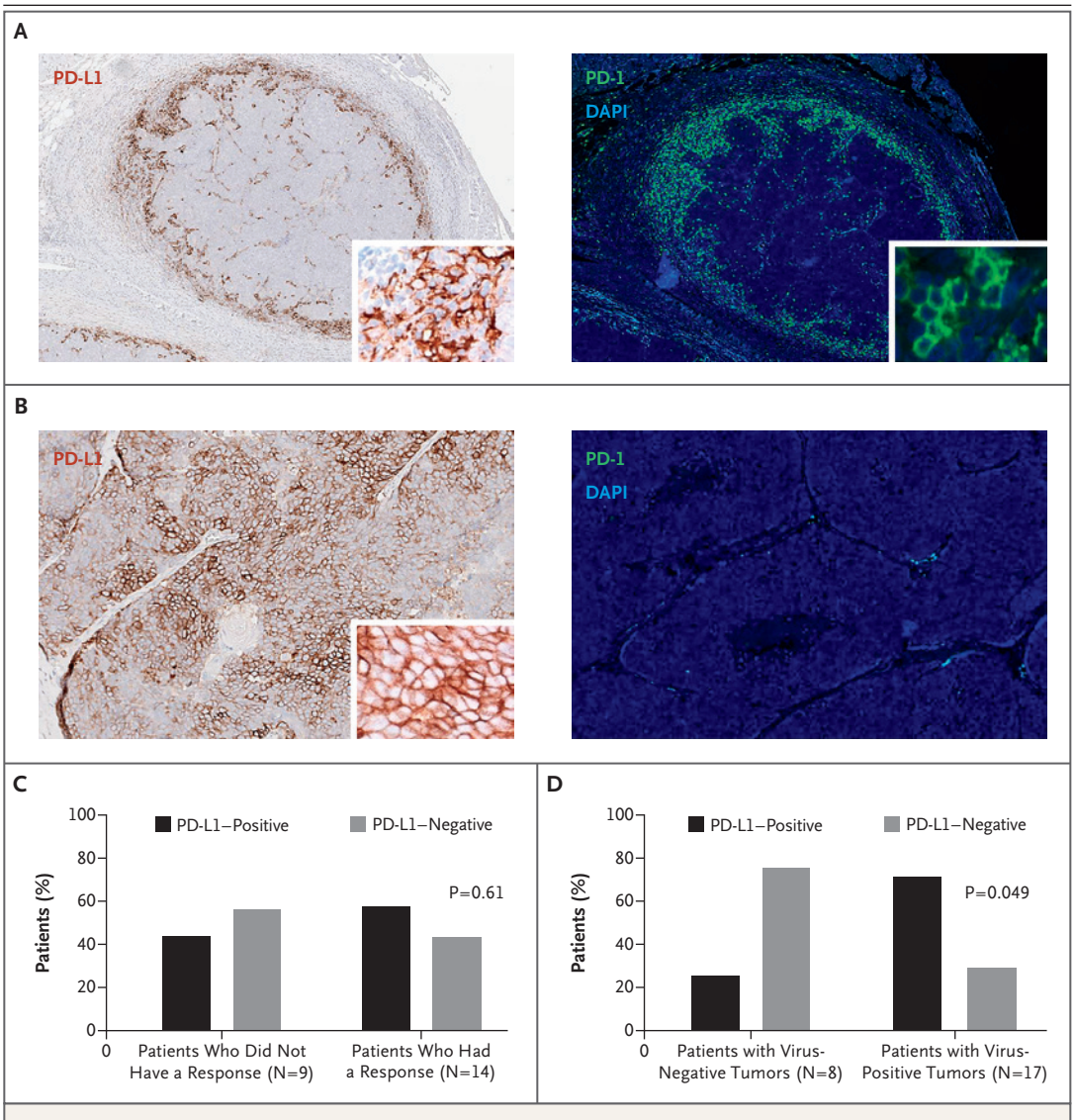


Figure 4. Expression of PD-1 and PD-L1 in Pretreatment Tumor Specimens, Detected by Immunohistochemical Testing.

Tumor-cell expression of PD-L1 (≥1% of tumor cells) was observed in 56% of tumors (14 of 25). Panels A and B show the results of chromogenic staining for PD-L1 (brown), immunofluorescent staining for PD-1 (green), and DAPI staining for nuclear DNA (blue). In most PD-L1+ tumors (11 of 14; 79%), PD-L1 expression was observed only in association with PD-1+ lymphoid infiltrates, typical of an "adaptive immune resistance" pattern (Panel A). One tumor (Panel B) showed broad, constitutive tumor-cell expression of PD-L1 that was independent of lymphoid infiltrates, with only a small focal area (not shown) of adaptive PD-L1 expression at the tumor periphery. The remaining two PD-L1+ tumors showed geographic areas of both constitutive and adaptive patterns of PD-L1 expression (not shown). As shown in Panel C, no significant association was observed between pretreatment tumor PD-L1 expression and response to pembrolizumab according to RECIST, version 1.1, among the 23 patients included in this analysis (P=0.61 by unconditional exact test on a two-by-two contingency table). Of the 3 patients who were not included in this analysis, 1 had not yet undergone a response evaluation, 1 had an unconfirmed response, and 1 had a stained tumor specimen that was technically inadequate. Samples were considered to be PD-L1–positive if at least 1% of tumor cells expressed PD-L1. Panel D shows the correlation of tumor MCPyV status with PD-L1 expression. A total of 71% of virus-positive tumors also showed PD-L1 expression on tumor cells; in contrast, only 25% of virus-negative tumors were positive for PD-L1 (P=0.049 by unconditional exact test). Only 25 of the 26 patients were included in this analysis, because 1 patient had a stained tumor specimen that was technically inadequate.

treated Merkel-cell carcinoma, an orphan disease for which available systemic cancer therapies do not meaningfully extend survival. In this study, response to pembrolizumab did not correlate with PD-L1 expression, a finding that contrasts with reports on some other cancer types. This may be because the response rate is relatively high and thus larger numbers of patients may be required to discern the discriminatory capacity of this test, although technical factors such as tumor sampling error and the use of archival tissues may also play a role. Ongoing studies correlating other features of the tumor microenvironment with clinical outcomes of anti–PD-1 or anti–PD-L1 therapy — examining the expression of additional immune checkpoints; the composition, density, and geography of T-cell infiltrates; gene-expression profiles; and single-variable vs. multiplex analyses — are anticipated to reveal more specific and powerful predictors. As of now, none of the predictive tests are sufficiently robust to be used in clinical decision making regarding whether to use or not to use PD-1 blockers in Merkel-cell carcinoma.

In the current study of pembrolizumab therapy in Merkel-cell carcinoma, we observed a 56% objective response rate. Tumor regressions occurred in multiple organ sites and in patients with bulky disease. Regressions appeared to be durable within an observation period of up to 9.7 months after initial documentation of a response. Twelve of 14 confirmed responses were ongoing at the time of analysis, and the estimated rate of progression-free survival at 6 months was 67%. Although additional experience with longer follow-up and larger patient cohorts is needed, these early findings compare favorably with results for standard chemotherapy regimens for this tumor,[7,39] for which retrospective studies show a median progression-free survival of approximately 3 months, with progressive disease developing in 90% of patients within 10 months.[6] Pembrolizumab was associated with previously described toxic effects in the relatively elderly patient population included in this study (median age, 68 years); we observed a 15% rate of grade 3 or 4 adverse events that were managed by discontinuation of pembrolizumab and initiation of glucocorticoid treatment as needed, without clear adverse effects on the magnitude or duration of tumor response.

We observed responses to anti–PD-1 in both MCPyV-positive and MCPyV-negative Merkel-cell carcinomas, which are reported to have markedly dichotomous mutational burdens. The median of 1121 mutations per exome reported in virus-negative Merkel-cell carcinoma exceeds the mutational burdens reported for other cancers that are responsive to anti–PD-1 or anti–PD-L1 therapies, including melanoma, squamous and nonsquamous non–small-cell lung cancers, and cancers of the bladder, head and neck, and kidney.[15,40] Conversely, the median of 12.5 mutations per exome observed in virus-positive Merkel-cell carcinoma is below those reported for tumor types that are poorly responsive to anti–PD-1, such as prostate and pancreatic cancer. Thus, potentially through distinct mechanisms (viral antigen expression or high tumor mutational load), both virus-positive and virus-negative Merkel-cell carcinomas appear to be immunogenic and susceptible to immune therapy by inhibition of the PD-1 pathway. Our current understanding of the mechanism of antitumor immunity induced by PD-1 blockade centers on the unleashing of an endogenous repertoire of T cells specific for neo-epitopes generated by a small subset of somatic mutations in the tumor — so-called mutation-associated neoantigens. However, because the mutational load of MCPyV-positive Merkel-cell carcinoma is so low, our findings, together with previous findings of MCPyV T-antigen–specific T cells in patients with virus-positive Merkel-cell carcinoma, suggest that antigens expressed by oncogenic viruses represent a distinct category of T-cell targets for immune checkpoint blockade.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

This paper is dedicated to the memory of Holbrook Kohrt, M.D., Ph.D., who contributed major elements of study design to this trial.

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Nghiem PT, Bhatia S, Lipson EJ, et al. PD-1 blockade with pembrolizumab in advanced Merkel-cell carcinoma. N Engl J Med. DOI: 10.1056/NEJMoa1603702

Supplementary Appendix for "PD-1 blockade with pembrolizumab in advanced Merkel cell carcinoma", by Nghiem et al.

Table of Contents to the Supplementary Appendix:

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II. SUPPLEMENTARY METHODS

Tumor Merkel Cell Polyomavirus (MCPyV) Status

Serology: Baseline serum samples from all patients were used to measure MCPyV small T-antigen oncoprotein antibody titers at Laboratory Medicine (University of Washington, Seattle, WA) as described.[1] Titers above 74 were considered positive.

Oncoprotein-specific T cells: All patients were low-resolution HLA genotyped to determine eligibility for CD8 T cell specific MCPyV peptide-MHC tetramer screening (Bloodworks Northwest, Seattle, WA). Pretreatment peripheral blood mononuclear cells (PBMCs) collected from patients with HLA-I types that corresponded to available MCPyV-specific tetramers (n=17) were tetramer stained to identify MCPyV-specific T cells, and analyzed by flow cytometry. Samples with >0.01% of CD8+ T cells co-staining with tetramers were considered positive. In addition, PBMCs from the first 12 patients with available pretreatment and week 12 post-treatment blood collections were stimulated with pools of MCPyV-specific peptides in a flow cytometry-based intracellular cytokine secretion assay (HIV Vaccine Trials Network, Seattle, WA). PBMCs that secreted interferon-gamma and/or IL-2 robustly (≥0.1% of CD8 T cells after background subtraction) were considered reactive to MCPyV.

Multispectral fluorescent immunohistochemistry

Position	Antibody	Clone (host)/Company	Dilution	Incubation	TSA dyes
1	PD-L1	SP142 (rabbit)/Spring Bio.	1:800	60 min	620
2	PD-1	EPR4877(2) (rabbit)/AbCam	1:1000	30 min	650
3	NSE	BBS/NC/VI-H14(mouse)/Dako	1:1000	60 min	570
4	CD68	PGM-1(mouse)/Dako	1:500	30 min	540
5	CD8	4B11(mouse)/AbD Ser	1:100	30 min	520
6	DAPI	Perkin Elmer Opal 7-color kit	2 drops/ml	5 min	

Formalin-fixed, paraffin-embedded tissues were cut in 4 um thick sections and placed on plus-charged slides. Slides were heated at 57°C overnight; then residual paraffin was removed using xylene, and tissue was rehydrated in a series of graded alcohols to distilled water. Antigen retrieval was performed using Tris-EDTA buffer and microwave treatment. Slides were washed and blocking was performed with 3% H2O2 blocking solution followed by Dako antibody diluent. The first primary antibody, i.e., Position 1 in the table above, was then applied and allowed to incubate. Opal polymer HRP Ms + Rb (Perkin Elmer, Hopkington, MA) was used as the secondary antibody. The slides were washed and the

tyramide signal amplification (TSA)-dye (Opal 7 color kit, Perkin Elmer, Hopkington, MA) for Position 1 was applied. Slides were then microwaved to strip the primary and secondary antibodies, washed, and blocked again using blocking solution. The second primary antibody, i.e., Position 2, was applied, and the process was repeated through Position 6, where DAPI was applied, rather than another primary antibody. After unbound DAPI was washed off, slides were coverslipped using Vectashield (Vector Laboratories, Burlingame, CA). In addition to the multiplex assay described above, a single color slide was generated for each antibody using an archival Merkel cell carcinoma case. Each single color control slide was compared to standard IHC methods for CD8, CD68, PD-L1, PD-1, and NSE (neuron-specific enolase).

Multiplexed and single color control slides were loaded onto the PerkinElmer Vectra automated multispectral microscope. Representative fields from the single color slides were imaged, and InForm Image Analysis software (Ver 2.1) was used to generate a spectral library for unmixing. Index cases stained using the multiplex method were then imaged. Channels were unmixed using the spectral library, and tissues and cells were segmented and scored.

III. SUPPLEMENTARY FIGURES

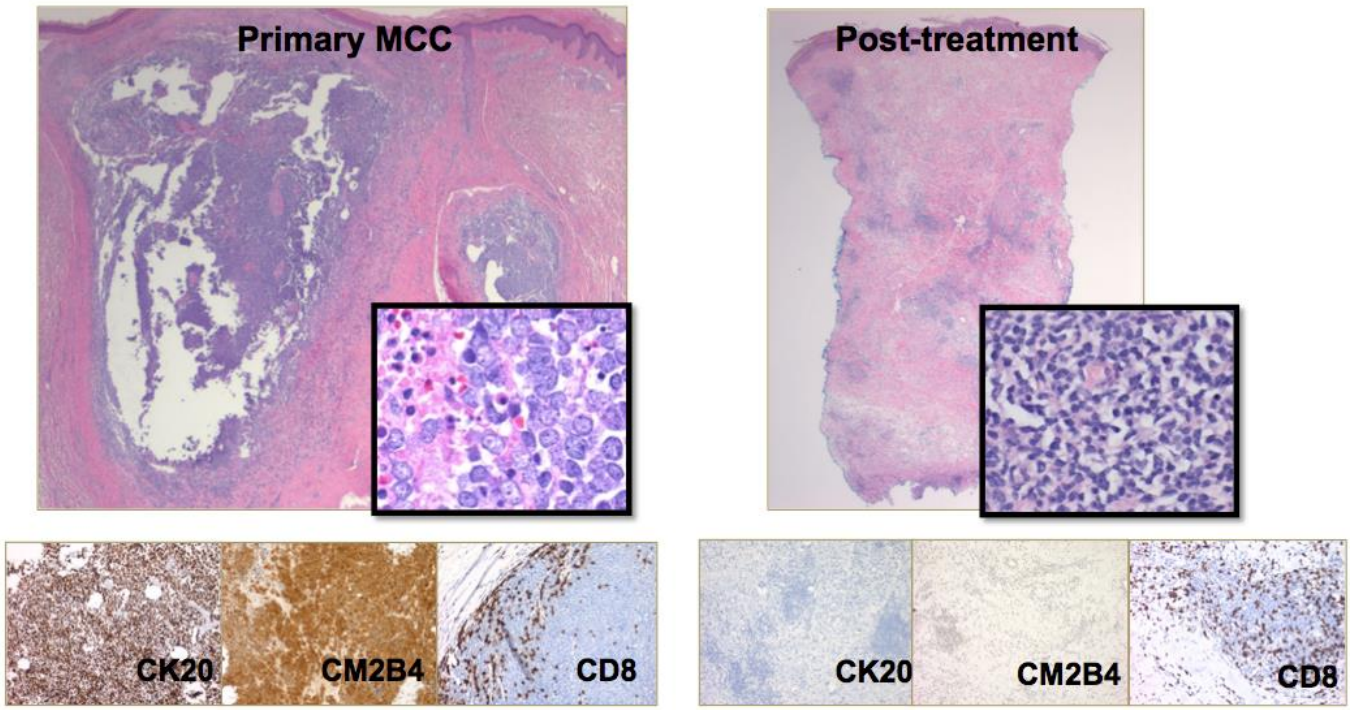

Figure S1: Pre- and on-treatment biopsy analysis in a patient responding to anti-PD-1

Biopsies of the primary Merkel cell carcinoma (MCC) lesion before anti-PD-1 therapy (left panels), and an adjacent subcutaneous metastasis regressing 3 weeks after initiating therapy (right panels) from the responding patient represented in **Figure 3**. Top panels, H&E stain (showing typical "salt & pepper" chromatin of MCC in primary lesion, and lymphoid infiltrates in post-treatment sample). Immunohistochemistry in lower panels shows no evidence of residual tumor cells and a robust CD8 infiltrate in post-treatment biopsy. CK20, cytokeratin marker for MCC; CM2B4, marker for MCPyV large T-antigen [2,3]; CD8, cytolytic T cell subset.

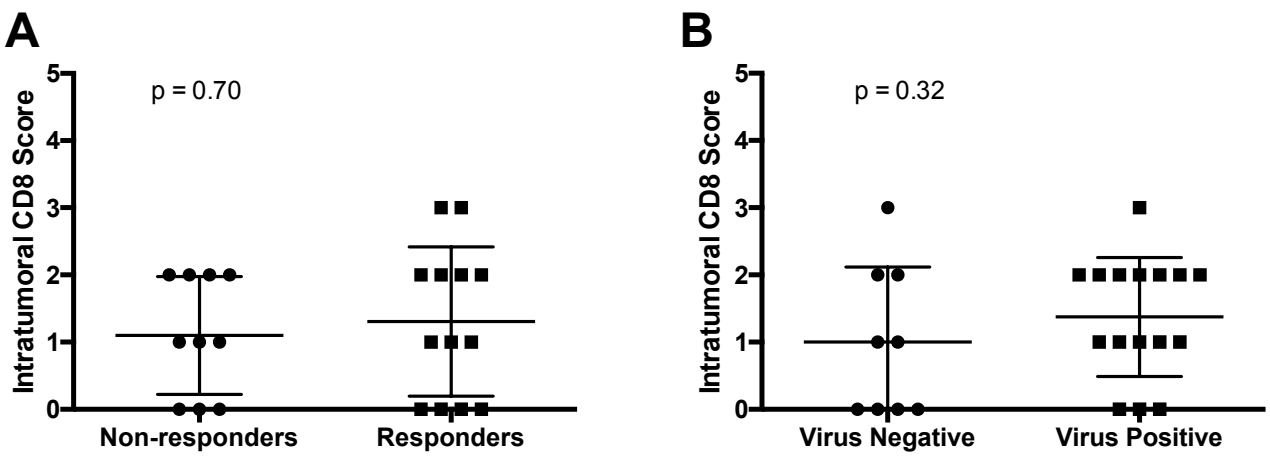

Figure S2: Pretreatment intratumoral CD8+ lymphocyte infiltration does not correlate with response to pembrolizumab or tumor viral status

Pretreatment tumor biopsy samples were obtained and analyzed. The period between the pretreatment tumor biopsy and treatment initiation ranged from 7 days to 8.4 years (median 5.2 months). Intratumoral (IT) CD8+ cells were defined as those surrounded completely by tumor cells without direct contact with stroma, as described previously. IT CD8+ infiltration was scored semi-quantitatively on 25 tumors with evaluable staining, on a 0-5 scale: 0 = no IT CD8+ cells; 1= 1-179 IT CD8+ cells/mm^2; 2=180-433 IT CD8+ cells/mm^2; 3= 434-582 IT CD8+ cells/mm^2; 4= 583-731 IT CD8+ cells/mm^2; 5 = ≥732 IT CD8+ cells/mm.[2,4] (**A**) IT CD8+ infiltration did not correlate with response (complete and partial responses) to pembrolizumab (n = 23; three patients were excluded because one patient did not yet have a response evaluation, one patient had an unconfirmed response, and one patient did not have sufficient tumor for CD8 interpretation). The mean score (on the 0-5 point scale) among responders was 1.3 versus 1.10 for non-responders; p = 0.70 by Mann-Whitney U test. (**B**) IT CD8+ infiltration did not correlate with viral status (n = 25 from all tumors with interpretable CD8 IHC; mean score of 1.38 in virus-positive vs 1.00 in virus-negative tumors; p = 0.32 by Mann-Whitney U test).

IV. SUPPLEMENTARY TABLES

Table S1: Adverse events

System organ class/ preferred term	All grades (1-4)** (no. patients, %)	Grades 3-4** (no. patients, %)
Total subjects with drug-related* adverse events	**20 (77%)**	**4 (15%)**
Blood and lymphatic system disorders	**3 (12%)**	
Anaemia	1 (3.8%)	
Leukocytosis	1 (3.8%)	
Microcytic anaemia	1 (3.8%)	
Thrombocytopenia	1 (3.8%)	
Cardiac disorders	**1 (3.8%)**	**1 (3.8%)**
Acute myocardial infarction	1 (3.8%)	1 (3.8%)
Myocarditis	1 (3.8%)	1 (3.8%)
Ventricular arrhythmia	1 (3.8%)	1 (3.8%)
Ventricular tachycardia	1 (3.8%)	1 (3.8%)
Atrial fibrillation	1 (3.8%)	
Bundle branch block left	1 (3.8%)	
Eye disorders	**2 (7.7%)**	
Eyelid ptosis	1 (3.8%)	
Ophthalmoplegia	1 (3.8%)	
Vision blurred	1 (3.8%)	
Gastrointestinal disorders	**4 (15%)**	**1 (3.8%)**
Small intestinal haemorrhage	1 (3.8%)	1 (3.8%)
Diarrhoea	1 (3.8%)	
Dry mouth	1 (3.8%)	
Nausea	1 (3.8%)	
General disorders and administration site conditions	**13 (50%)**	
Fatigue	12 (46%)	
Chills	2 (7.7%)	
Asthenia	1 (3.8%)	
Feeling hot	1 (3.8%)	
Local swelling	1 (3.8%)	
Nodule	1 (3.8%)	
Infections and infestations	**1 (3.8%)**	
Oral candidiasis	1 (3.8%)	
Injury, poisoning and procedural complications	**1 (3.8%)**	
Fall	1 (3.8%)	
Investigations	**6 (23%)**	**3 (12%)**
Aspartate aminotransferase increased	4 (15%)	3 (12%)
Alanine aminotransferase increased	2 (7.7%)	2 (7.7%)
Blood creatine phosphokinase increas	1 (3.8%)	1 (3.8%)
Blood alkaline phosphatase increased	1 (3.8%)	
Blood bilirubin increased	1 (3.8%)	
Blood corticotrophin decreased	1 (3.8%)	
Blood creatinine increased	1 (3.8%)	
Blood urine present	1 (3.8%)	
Weight decreased	1 (3.8%)	

Metabolism and nutrition disorders	**3 (12%)**	**2 (7.7%)**
Hyponatraemia	2 (7.7%)	2 (7.7%)
Hyperglycaemia	1 (3.8%)	1 (3.8%)
Decreased appetite	1 (3.8%)	
Malnutrition	1 (3.8%)	
Musculoskeletal and connective tissue disorders	**3 (12%)**	
Myalgia	2 (7.7%)	
Arthralgia	1 (3.8%)	
Neoplasms benign, malignant and unspecified (including cysts and polyps)	**2 (7.7%)**	**1 (3.8%)**
Tumour pain	2 (7.7%)	1 (3.8%)
Nervous system disorders	**4 (15%)**	**1 (3.8%)**
Encephalopathy	1 (3.8%)	1 (3.8%)
Cerebral ischaemia	1 (3.8%)	
Dizziness	1 (3.8%)	
Headache	1 (3.8%)	
Hypoaesthesia	1 (3.8%)	
Nystagmus	1 (3.8%)	
Paraesthesia	1 (3.8%)	
Sciatica	1 (3.8%)	
Psychiatric disorders	**1 (3.8%)**	
Delirium	1 (3.8%)	
Disorientation	1 (3.8%)	
Renal and urinary disorders	**3 (12%)**	**1 (3.8%)**
Renal failure acute	1 (3.8%)	1 (3.8%)
Proteinuria	1 (3.8%)	
Renal failure	1 (3.8%)	
Urinary retention	1 (3.8%)	
Reproductive system and breast disorders	**2 (7.7%)**	
Breast pain	1 (3.8%)	
Pruritus genital	1 (3.8%)	
Respiratory, thoracic and mediastinal disorders	**3 (12%)**	
Cough	2 (7.7%)	
Pneumonitis	1 (3.8%)	
Skin and subcutaneous tissue disorders	**4 (15%)**	
Alopecia	2 (7.7%)	
Itching scar	1 (3.8%)	
Pruritus	1 (3.8%)	
Rash	1 (3.8%)	
Uncoded	**1 (3.8%)**	**1 (3.8%)**
Acute Combined Systolic And Diastolic Heart Failure	1 (3.8%)	1 (3.8%)
Vascular disorders	**5 (19%)**	
Hypotension	2 (7.7%)	
Hot flush	1 (3.8%)	
Hypertension	1 (3.8%)	
Phlebitis	1 (3.8%)	

*Adverse events recorded as having a Definite, Probable or Possible association with the study drug were considered drug-related.
**A subject that experienced multiple occurrences of an adverse event was counted once at the maximum grade recorded.

V. REFERENCES

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Chapter 7

Immune correlates of response to PD-1 blockade in Merkel cell carcinoma

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Abstract:

Purpose: Merkel cell carcinoma (MCC) is an aggressive cancer associated with exposure to UV-mutagenesis and/or the Merkel cell polyomavirus (MCPyV). In a phase II study of pembrolizumab (anti-PD-1 monoclonal antibody) in patients with metastatic MCC, an overall response rate of 56% was observed. Correlative studies were performed to assess the MCPyV-specific B and T cell responses of these patients throughout therapeutic course, as well as to investigate potential biomarkers that may be predictive of response.

Experimental Methods: PBMC obtained pre- and post-therapy was assessed for presence of MCPyV-specific tetramer + T cells, reactivity to MCPyV-specific peptides, and T cell phenotype. MCPyV oncoprotein antibody titers were assessed throughout therapeutic course. Archival tumor material was obtained for all patients (n=26) and assessed by immunohistochemical staining and T cell receptor (TCR) sequencing.

Results: MCPyV-specific T cells were detected in 66% of HLA class I matched patients with virus-positive tumors but their presence was not associated with response to pembrolizumab. The fraction of all circulating CD8+ T cells expressing PD-1 at baseline was not associated with response to pembrolizumab. Circulating T cell reactivity to MCPyV peptides did not change after therapy in most patients; one patient with an impressive partial response to pembrolizumab had an increase in MCPyV-tetramer+ T cells that was associated with a robust increase in MCPyV-specific T cell function. Tumoral TCR clonality was associated with viral status, but not with response. MCPyV oncoprotein antibody titers were detectable in 58% of patients and decreased after effective therapy with pembrolizumab. Tumoral immunohistochemical markers (PD-L1 expression on immune cells or at tumor perimeter, PD-1 expression, and peritumoral CD8+ cells) were not associated with response to pembrolizumab.

Conclusions: No significant immune correlates of protection were found among analyses of pre-treatment archival tumor biopsies and pre- and post-treatment blood from metastatic MCC

patients receiving pembrolizumab. Further efforts assess the reactivity of immune cells in the tumor microenvironment are ongoing.

Introduction:

Merkel cell carcinoma is an aggressive neuroendocrine skin cancer. Approximately 80% of MCCs are driven by T-antigen oncoproteins of the Merkel cell polyomavirus (MCPyV), while 20% of 'virus-negative' MCCs are driven by UV-induced mutagenesis[1,2]. Both subtypes of MCC are immunogenic and can elicit MCC-specific CD8+ and CD4+ T cell responses that can be effective at fighting MCC[3](unpublished). In addition, approximately half of virus-positive patients produce serum antibodies against the MCPyV small T-antigen oncoprotein that can be used to track disease burden[4]. Despite the presence of these immune responses, MCCs develop and persist, implying local or systemic immune dysfunction. Indeed, we have found that MCC employs many immune escape mechanisms including upregulation of PD-1 on MCPyV-specific T cells[5] and expression of PD-L1 on tumor and immune cells[6,7]. These findings suggested that MCC would be particularly susceptible to treatment with immune-based therapies such as anti-PD-1 agents.

The prognosis of MCC is poor, and there are currently no FDA approved agents for the treatment of MCC. Approximately 1/3 of patients diagnosed with MCC will develop metastatic disease, with a median time from metastasis to death of only ~9 months[8]. Chemotherapy can elicit tumor shrinkage in approximate half of patients, but responses are not durable with a median time to progression of just 3 months[9]. Fortunately, the frontier of treatment for MCC is promising, due in large part to immune-based therapies such as immune checkpoint inhibitors. We have recently published the results of our Phase II clinical trial of pembrolizumab, an anti-PD-1 monoclonal antibody, for the treatment of metastatic MCC. We observed an objective response rate of 56% among all patients, with a similar response rate among both virus-negative and virus-positive patients[10]. Excitingly, these responses appear more durable than chemotherapy, with 67% of responding patients progression-free 6 months after starting pembrolizumab.

Several unresolved questions remain from the Nghiem, *et al* study. Expression of PD-L1, a ligand of PD-1, within tumors has been associated with response to PD-1 inhibitors in other cancers such as melanoma[11-14] but this association was not found in MCC. A biomarker to predict which MCC patients will respond to therapy is therefore still needed. In addition, MCC offers an ideal model system in which to track and assess cancer-specific T and B cell responses throughout therapeutic course because the majority of MCC tumors are driven by a viral oncoprotein with a known amino acid sequence. Studying the immune response against MCPyV associated MCCs may provide insight into the mechanisms of these agents. These studies may also elucidate which agents may best be combined with PD-1 inhibitors to improve therapeutic efficacy in MCC. With these aims, the current study was undertaken to thoroughly examine any correlates of response among tumor and blood samples from these clinical trial patients.

Methods:

Patients and patient samples: Twenty-six patients were enrolled in this phase 2, single arm, Simon two-stage, multi-center study. All patients provided written informed consent and the study was conducted in accordance with the Declaration of Helsinki. Eligible patients were at least 18 years old with distant metastatic or recurrent locoregional MCC not amenable to definitive surgery or radiation therapy, measurable disease per RECIST 1.1, Eastern Cooperative Oncology Group (EGOG) performance status of ≤ 1 (on scale 0-5, lower score indicates less disability), and normal organ and bone marrow function. Blood samples were drawn for correlative laboratory analyses immediately pre-treatment, 12 weeks after starting therapy, and at 9-week intervals thereafter. Peripheral blood mononuclear cells (PBMC) were cryopreserved after routine Ficoll preparation by specimen processing facility at the CITN.

MCPyV-specific Tetramer Staining: All patients were low-resolution, polymerase chain reaction (PCR), HLA class I genotyped to determine eligibility for CD8 T cell specific MCPyV-tetramer screening (Bloodworks Northwest, Seattle, WA). PBMC collected from patients with HLA class I types that corresponded to available MCPyV-specific tetramers (A*02:01, A*24:02, B*07:02, B*35:02, or B*37:01; n=17 patients) were analyzed with appropriate tetramers. At least 2 million PBMC acquired at both baseline and 12 weeks after starting therapy were stained with anti-CD8-FITC antibody (Clone 3B5, Life Technologies), 7-AAD viability dye (BioLegend), and appropriate APC or PE-labeled tetramers (Immune Monitoring Lab, FHCRC) and data collected on a FACSAriaII (BD). FlowJo version 10.0.8 (TreeStar) was used to for data analysis and determination of the percentage of live cells in the tetramer, CD8, double positive region. Samples with >0.01% of CD8+ T cells co-staining with tetramers were considered positive.

Circulating T cell phenotype and responsiveness to MCPyV peptides: Pre-treatment PBMC (n=12) and post-treatment PBMC obtained 12 weeks (n=10) or 21 weeks (n=1) after initiating

therapy were submitted for T cell phenotyping and intracellular cytokine secretion assays (HIV Vaccine Trials Network (HVTN), Seattle WA). After an overnight rest, at least 1 million viable PBMC per condition were interrogated with pool of 13aa-long peptides from each of Pools 1, 2, 3 and 10 (~25 peptides each) corresponding to the persistently expressed region of MCPyV, as well as positive (CMV) and negative (DMSO) controls. Cells were stimulated for 6 hours at 37°C with relevant peptides and 1 ug/ml costimulatory antibodies CD28 and CD49d (Becton Dickson (BD) Biosciences) in the presence of 10 ug/ml Brefeldin A (Sigma). Cells were stained for a panel of surface markers including: CD3, CD4, CD8, PD-1, CD45RA, CCR7, CD40L, and for intracellular cytokines: IFN-γ, IL-2, TNFα, IL-21, and IL-4. Data was collected by flow cytometry on a LSRII and analyzed with FlowJo version 8.8.7 (TreeStar). Responsiveness to MCPyV peptides was based on IFN-γ and IL-2 expression by CD8 and CD4 T cells. T cell effector phenotype was based on CD45RA and CCR7 expression by CD8 and CD4 T cells.

Phenotyping of MCPyV-specific tetramer + T cells: Cryopreserved PBMC (~10 million PBMC/vial) were thawed and immediately stained with Live/Dead Aqua Viability dye (LifeTechnologies). Cells were pretreated with 100 nM dasatanib for 10 minutes at 37°C before addition of APC-conjugated MCPyV-specific tetramers or a CMV-specific tetramer conjugated to PE (HLA-A*24/MCPyV.LT-92-101, HLA-A*02/MCPyV.CT-15-23, HLA-A*24/QYDPVAALF; Immune Monitoring Lab, FHCRC Seattle, WA). Cells were incubated in blocking buffer (FACS buffer [PBS+ 0.1% BSA + 0.1% NaN_3] + 4% normal mouse serum (Santa Cruz Biotechnologies) and 0.2% Human IgG (Pierce Biotechnology). Cells were stained for 30 min at 4°C with: CD8-BUV395 (BD); CD45RO-BV605 (BD); CCR7-BV711 (Biolegend), CD4-, CD19-, and CD14-BV785 for dump gating (Biolegend); TIM-3-AF750 (R&D); PD-1-BV421 (Biolegend); LAG3-FITC (Enzo Life Sciences); CD57-BV570 (Biolegend); 2B4-PerCP-Cy5.5 (Biolegend); and CD160-PE-Cy7 (Biolegend). Cells were washed and at least one million events were collected on a

LSRII flow cytometer (BD) and analyzed using FlowJo version 10.0.8 (Tree Star, Inc). Fluorescence minus-one controls were used to determine positivity for inhibitory markers.

T cell receptor sequencing and analysis: Whole tumor sequencing: Samples were sequenced from all 26 patients pre-treatment tumors and 4 patients post-treatment tumors. Tumor material consisted of 25 micron molecular curls (n=12) or scraped sections from ~five, 4 micron slides (n=18) from formalin-fixed, paraffin embedded (FFPE) blocks. Samples were submitted to Adaptive Biotechnologies for genomic DNA extraction, TCRβ sequencing and normalization. T cell fraction was determined by estimating the fraction of cells with productive TCR rearrangements out of all nucleated sample cells. T cell receptor clonality: Shannon entropy was calculated on the estimated number of genomes (≥2) of all productive TCRs and normalized by dividing by the log2 of unique productive sequences in each sample. Clonality was calculated as 1- normalized entropy.

Serology: Baseline serum samples from all patients were used to measure MCPyV small T-Antigen oncoprotein antibody titers at Laboratory Medicine (University of Washington, Seattle, WA) as described[4]. Titers above 74 were considered positive.

Immunohistochemical tumor analysis: PD-L1 and PD-1 staining was performed at Merck Research Laboratories (Palo Alto, CA) on formalin-fixed paraffin-embedded (FFPE) tissue sections. Slides were subjected to heat-induced epitope retrieval and blocking of endogenous peroxidase prior to incubation with primary antibody (anti-PD-L1 mAb clone 22C3, Merck Research Laboratories, Palo Alto, CA or anti-PD-1 goat polyclonal antibody, R&D Systems, Minneapolis, MN). Antigen-antibody binding was visualized using 3,3' diaminobenzidine (Dako, Carpinteria, CA) for PD-L1, or AlexaFluor 488 (Invitrogen, Grand Island, NY) for PD-1. PD-1

expression was scored for prevalence of positive cells on a semiquantitative 0-5 scale. PD-L1 expression on immune cells was scored on a semiquanititative 0 (absent) to 3 (multiple areas of infiltratation or diffuse infiltration and/or dense immune cells along large stretches of perimeter) scale. Immune cell scores were binned with 0-1 = low PD-L1 expression and 2-3 = high PD-L1 expression. PD-L1 positivity at the tumor perimeter was measured by total % of perimeter staining, with 0-4% binned as low PD-L1 expression and >5% binned as high PD-L1 expression.

Tumor sections were also stained with anti-CD8 (Dako, clone 144B) to detect cytolytic T cells. Peritumoral CD8+ T cells (completely surrounded by tumor and not abutting stroma) were scored by a dermatopathologist who was blinded to patient characteristics, on a scale from 0 (absent) to 5 (brisk/band-like peritumoral lymphocytic infiltrate; CD8+ cells make up >50% of peritumoral lymphocytic infiltrate).

Results:

Circulating immune cells and patient response to pembrolizumab

We asked whether the presence of B or T cell reactivities to MCPyV correlated with patient response to pembrolizumab. Data for patient's tumor viral status, serum positivity for oncoprotein-specific antibodies, and presence of MCPyV-specific tetramer+ CD8+ T cells is tabulated along with patients' response according to RECIST 1.1 in **Table 1**. B and T cell reactivities to MCPyV were found only in patients with virus-positive tumors, but patients with virus-positive tumors often lack these responses as previously observed[4,5].

MCPyV-specific Tetramer + T cells

Tumor-specific CD8+ T cells are likely the crucial mediator of tumor shrinkage seen in 56% of patients in response to pembrolizumab. We therefore studied the phenotype, function, and frequency of MCPyV-specific T cells, using our established HLA-I MCPyV-specific tetramers. Pre- and post-treatment PBMC from all patients with an HLA-I type corresponding to our 5 MCPyV-specific tetramers, regardless of tumor MCPyV status, was screened with the relevant tetramers.

Tetramer + T cells were detected among pre-treatment PBMC in 5 of 17 patients with appropriate HLA-I types, 9 of whom were later determined to have MCPyV+ tumors. Post-therapy, a nascent tetramer + population became detectable in one patient. We did not observe any correlation between the presence of MCPyV-specific T cells at baseline and response to pembrolizumab (**Table 1**).

MCPyV-specific T cells, previously also found only in patients with MCPyV+ tumors, have been found to track with disease burden. However, we hypothesized that activation of the immune system with pembrolizumab could induce proliferation of antigen-specific cells. We therefore assessed the frequency of tetramer + T cells throughout therapeutic course (n= 5 patients). The frequency of tetramer + T cells did not consistently fluctuate over time in the

patients studied, regardless of response status (**Figure 1**). This may reflect a combination of both PD-1 stimulation of MCPyV-specific T cells to proliferate and decreased antigen load.

MCPyV-specific T cell responses

We next wanted to study the phenotype of T cells in patients who did and did not respond to pembrolizumab, to understand whether T cells became more responsive to stimulation with MCPyV-specific peptides after treatment with pembrolizumab. Pre- and post-treatment PBMC from 11 patients (remaining patients being tested), in addition to pre-treatment PBMC only from 1 patient, was stimulated with pools of MCPyV-specific peptides corresponding to the persistently expressed region of MCPyV and cytokine production was assessed via flow cytometry. Patients were selected without knowledge of their tumor viral status. No responses by CD4+ T cells were detected from any PBMC sample obtained pre- or post-treatment.

In pre- or post- PBMC from seven patients with virus-positive tumors, only two patients had CD8+ T cell responses (as measured by IFNγ and/or IL-2 secretion) to MCPyV-specific peptides. One patient had a response to one peptide pool; this response remained at constant frequency in the post-treatment sample (**Figure 2A**). The second patient had responses to 2 peptide pools, and these responses were ~15x higher in the post- vs pre- treatment PBMC. This was accompanied by an increase of ~7x in the tetramer + T cell population using a tetramer containing an epitope found within one of the peptide pools the patient responded to (**Figure 2B**). We therefore hypothesize that an increase in frequency of MCPyV-specific T cells, rather than a gain of function, was predominantly responsible for the increased frequency of IFNγ secreting T cells in this patient after pembrolizumab.

T cells responding to both peptide pools were polyfunctional (expressed TNFα and/or IL-2 in addition to IFNγ), displayed an effector memory phenotype (all were CD45RA-/CCR7-), and expressed PD-1 (56.7% and 76.1%, **Figure 2C**). Excitingly, this patient had a very impressive

partial response to pembrolizumab, as visualized by comparing CT scans obtained at baseline and 12 weeks after initiating therapy that show a reduction of necrotic liver masses from 13 cm to 6.4 cm at longest diameter (**Figure 2D**). This patient later developed metastatic disease, which may have contributed to the expansion of these MCPyV-specific T cells in the circulation.

Phenotype of circulating cells

We assessed the expression of PD-1 on all circulating CD8 T cells from pre- and post-treatment PBMC (n=11; remaining patients being tested). In 10 of 11 patients, the % of PD-1 positivity on CD8 T cells decreases post-therapy, by a median of 36% (**Figure 3A**). There was no association between PD-1 expression on all CD8 T cells pre-treatment and response to pembrolizumab (**Figure 3B**, p= 0.58 by Mann Whitney). The frequencies of different CD8 T cell subtypes within each patient (Naïve, central memory, effector memory, or terminally differentiated) did not significantly change after therapy. There is a trend toward an increased % of CD8 T cells that have an effector memory phenotype among patients with progressive disease (**Figure 3C**).

Phenotype of circulating MCPyV-tetramer+ T cells

We determined the expression of numerous inhibitory receptors and other phenotypic markers on the surface of MCPyV-specific T cells using a tetramer-based multicolor flow cytometry panel. Only two patients, one who had a complete response to pembrolizumab and one who immediately progressed, had sufficient quantities of tetramer + T cells to reliably allow such analysis.

Comparing the level of inhibitory markers on MCPyV-specific T cells at baseline from these two patients, the % of PD-1 positivity and the mean fluorescence intensity of PD-1 expression is greater on the patient who did not respond to therapy (**Figure 4A-B**).

We analyzed the phenotype of MCPyV- and CMV- specific T cells throughout therapeutic course for the patient with complete response, using CMV-specific T cells as an internal control. After therapy, the expression of PD-1 decreases on both MCPyV- and CMV- specific T cells, but expression of another inhibitory receptor 2B4 decreases only on MCPyV-specific T cells (**Figure 4C**).

TCR repertoire of pretreatment tumors

We sequenced the complementarity determining region 3 (CDR3) region of *TRB* of T cells from FFPE sections of all pretreatment tumors (n=26). We calculated the *TRB* clonality of each tumor analyzed. Increased clonality of the immune infiltrate within tumors is thought to represent an enrichment of cancer antigen-specific T cells and has been associated with improved response to pembrolizumab in patients with metastatic melanoma[14]. There was no significant difference in TCR clonality between patients who did and did not respond to pembrolizumab (**Figure 5A,** p=0.72 by Mann-Whitney test). However, TCR clonality is significantly increased in patients with virus-positive MCCs (n=15) compared to those with virus-negative MCCs (**Figure 5B,** n=10, p=0.0009 by Mann-Whitney test), which likely represents the expansion of MCPyV-specific T cells in virus-positive tumors.

We then used this TCR sequencing data to calculate the fraction of each pretreatment tumor comprised of T cells, termed the 'T cell fraction'. We hypothesized that patients whose tumors had an increased T cell fraction would be more likely to respond to pembrolizumab. However, the T cell fraction was not significantly different between patients who did and did not respond to pembrolizumab (**Figure 5C,** p=0.89 by Mann-Whitney test). We did observe a trend for increased T cell fraction among virus-positive tumors (**Figure 5D**, p=0.095 by Mann-Whitney test).

MCPyV-specific B cell responses track with response to pembrolizumab

We measured B cell reactivities to MCPyV by measuring the titer of antibodies specific for the small T-Antigen oncoprotein with serum of all patients. These antibodies have previously been found to be highly specific for MCPyV+ MCC patients and titer has been shown to rise and fall in tandem with disease burden[4]. Oncoprotein antibodies were detected in pre-treatment serum from 15 of 26 patients (58%). There was no association between presence of oncoprotein antibodies and response to pembrolizumab. At least one post-treatment serum sample was analyzed for oncoprotein titer from all patients possible (n=18), regardless of their titer at baseline. Out of 7 patients who had been seronegative at baseline, none developed oncoprotein antibodies after treatment with pembrolizumab.

Oncoprotein antibody titer was tracked over time in 11 seropositive patients. Consistent with previous observations where titer decreases as tumor burden decreases, titer generally decreased after therapy in the 8 seropositive patients who experienced clinical responses to pembrolizumab (**Figure 6A,** titer over time and **6B,** fold change from baseline). In one patient who experienced a pathological complete response after just three weeks of therapy, oncoprotein antibody titer increased by two-fold over baseline. This is suggestive of immune activation in response to pembrolizumab. One patient with an initial partial response yet rising titer eventually developed progressive disease (**Figure 6A, middle panel**). For three patients who did not respond to pembrolizumab, titer either increased (in progressing patient) or remained constant (in one stable and one progressing patient. In summary, oncoprotein antibodies appear to track with disease burden but are not associated with improved response to pembrolizumab. Titers obtained more closely after initiation of therapy would likely provide more insight into any contribution of B cell responses.

Pathologic features of pre-treatment tumors do not correlate with clinical response

PD-L1, a ligand for PD-1, is expressed on both tumor and immune cells in MCC[6,7]. We were able to evaluate PD-L1 staining on pretreatment tumor biopsies from 25 of 26 patients.

Out of those 25 patients, 24 patients had at least one radiological post-therapy response evaluation. We previously published that expression of PD-L1 on tumor cells did not correlate with patient response to pembrolizumab[10]. In addition, we scored PD-L1 expression on both immune cells and at the interface between tumor and stroma (hereafter, the tumor perimeter). We observed that the degree of peritumoral CD8 T cell infiltration was significantly higher in tumors expressing PD-L1 on tumor cells, immune cells, or perimeter at baseline (data not shown), indicative of an adaptive immune response. However, in accordance to our previous findings, there was no correlation between the patients with low or high expression of PD-L1 on immune cells and response to pembrolizumab (**Figure 7A**, p=0.60 by unconditional exact test). Similarly, there was no association between the percentage of tumor perimeter expressing PD-L1 and clinical response to pembrolizumab (**Figure 7B**, p=1 by unconditional exact test).

In addition, we quantified the expression of PD-1 on 25 of 26 evaluable pre-treatment tumors using a 0-5 semi-quantitative scale. Of the 24 patients with a response evaluation, there was no difference between mean PD-1 expression among responders (n= 15) and non-responders (n=9, **Figure 7C**, p=0.71 by Mann Whitney test).

Lastly, we studied the expression of CD8+ T cells in pre-treatment tumors. We previously found that there was no significant association between mean intratumoral CD8+ score and response to pembrolizumab[10]. Additionally, we quantified the peritumoral CD8+ T cells using a 0-5 scale, and found no significant difference between the mean score among responding vs non-responding patients (**Figure 7D**, p=0.48 by Mann Whitney test).

Pathologic features of pre-treatment tumors do not correlate with tumor viral status

PD-L1 expression on immune cells and the tumor perimeter was evaluable for 25 out of 26 pre-treatment tumors. In accordance to our previous findings, we observed that patients with virus-positive tumors were more likely to have high PD-L1 expression on immune cells (**Figure 8A**, p=0.049 by unconditional exact test). There was a similar but not-statistically significant

trend between the percentage of tumor perimeter expressing PD-L1 and patient tumor viral status (**Figure 8B**, p=0.13 by unconditional exact test).

We quantified the expression of PD-1 on 25 of 26 evaluable pre-treatment tumors using a 0-5 semi-quantitative scale. Of the 24 patients with a response evaluation, there was no difference between mean PD-1 expression among patients with virus-negative tumors (n= 8) and patients with virus-positive tumors (n=17, **Figure 8C**, p=0.86 by Mann Whitney test).

Additionally, we quantified the presence of peritumoral CD8+ T cells in pre-treatment tumors, and found a trend toward increased CD8+ T cells among patients with virus-positive tumors (**Figure 8D,** p=0.059 by Mann Whitney test).

Discussion:

First-line therapy with the PD-1 blocking agent pembrolizumab resulted in an objective response rate of the majority (56%) of patients with metastatic Merkel cell carcinoma[10]. The objective of the current study was to identify and characterize a biomarker for response based on achieved tumor tissue and blood samples from these trial patients. In addition, we hypothesized that our ability to uniquely study cancer-antigen-specific B and T cell responses from many of these patients (those with MCPyV-positive tumors) may provide insight into the mechanism of action of pembrolizumab on the immune system in MCC patients. As a substantial portion of MCC patients do not respond to pembrolizumab and/or progress on therapy, these studies were also aimed at uncovering which additional therapies (checkpoint inhibitors or other immune-stimulating agents) might be best combined with PD-1 inhibitors to elicit tumor regressions in this subset of patients.

We have extensively analyzed multiple parameters within pre-treatment tumors via immunohistochemistry. Despite uncovering evidence of adaptive immune resistance (ie, similar patterns of PD-L1 and PD-1 expression, as well as correlations between PD-L1 expression and peritumoral CD8 T cells), as previously noted in MCC[6] and previously found to correlate with response to PD-1 blockade in melanoma[14], we have not uncovered any biomarkers that reliably correlate with patient response to pembrolizumab. In addition, we did not find any correlation between the presence of either circulating B and T cell responses to MCPyV-specific antigens and patient response to pembrolizumab.

These results contrast with studies in many cancers showing that patients with PD-L1+ tumors are more likely to respond to PD-1 blockade[11-14]. The inherent heterogeneity of tumor samples, as has been observed for PD-L1 expression even within different tumors from the same patient[11], may have impacted these results. In addition, our sample size is significantly smaller than most other trials. Study of additional MCC patients now being enrolled in the expansion cohort may provide more power to uncover any putative association. In addition, we

were limited in our ability to assess changes in the tumor microenvironment from pre- to post-therapy by the limited availability of post-treatment samples, obtained for only four of 26 patients (15%) in this trial. Additional post-treatment tumor biopsies from the expansion trial may facilitate our understanding of the tumor microenvironment throughout therapeutic course in responding and non-responding patients.

A previous study in melanoma found that increased TCR clonality within pretreatment tumors was associated with response to pembrolizumab, but this finding was not replicated in our study of an equivalent number of MCC patients. This may be due to variability within tumor specimens, as discussed above, or may truly indicate that TCR clonality and response to PD-1 are not associated in MCC. Interestingly, we did see a strong association between TCR clonality and virus-positive tumor status, which likely reflects the expansion of MCPyV-specific clones within virus-positive tumors.

Both MCPyV-specific B cell reactivity (as measured by serum oncoprotein antibody titer) and the frequency of MCPyV-specific T cells decreased over time in patients with decreasing tumor burden, as would be expected by previous findings[4,5]. In an analogous study of cancer-antigen specific T cells in non-small cell lung cancer, neoantigen-specific CD8+ T cell frequency increased 3 weeks after initiation of PD-1 blockade, in parallel with tumor regression, and fell shortly thereafter[15]. Our analysis of MCPyV-specific T cell responses from PBMC acquired at 12 weeks post-treatment may have missed an early spike in T cell reactivity. Indeed, in one patient where serum obtained 3 weeks post-treatment was analyzed, oncoprotein antibody titer spiked to twice the level of baseline before steadily decreasing to less than 25% of baseline. Surprisingly, we infrequently observed circulating T cell responses after stimulation with MCPyV-specific peptides. Indeed, among 7 virus-positive patients (all of whom responded to therapy), PBMC from only one patient displayed increased MCPyV-specific reactivities after therapy with pembrolizumab. One potential explanation is that MCPyV-specific T cells may have increased effector function, but may have migrated from the circulation into tumors in these

responding patients. In addition, the observed reactivity in one patient with an initial PR but eventual progression may be due to the development of new lesions that were detected shortly after the initial partial response.

Many opportunities exist to further assess the pre-existing immunity among patients who do and do not respond to PD-1 blockade, including transcriptome analysis of MCPyV-specific T cells and additional immunohistochemical analysis such as Ki67 and granzyme, etc, to measure activity, and not just number, of tumoral CD8+ T cells. In addition, we have anecdotal evidence that ipilimumab can be effective when added to the therapeutic regimen of a patient who did not respond to pembrolizumab and/or adoptive T cell therapy (unpublished observations). Detailed molecular study of samples from patients who respond to ipilimumab after progressing on PD-1 blockade may reveal biomarkers indicative of which patients should be treated with this combination. We have also observed upregulation of PD-L1 expression in patients treated with intratumoral glucopyranosyl lipid antigen (GLA), a TLR-4 agonist, implying that PD-1 blockade may synergize with this therapy. Lastly, a great opportunity exists to uncover neoantigen-specific CD8+ T cells hypothesized to mediate responses to PD-1 therapy in MCPyV-negative patients, and to then assess for similarities and differences between the responses of MCPyV- versus neoantigen-specific T cells to therapy.

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Figure Legends:

Figure 1: MCPyV-specific tetramer + T cells were detected in pre-treatment PBMC in 5 of 17 patients with appropriate HLA-I types. An additional tetramer + population became detectable in one patient post-therapy. The frequency of tetramer + T cells increased after therapy in patients with a partial response (black, n=3) and decreased or remained constant in patients with a complete response (grey, n=2). Post-treatment PBMC was not available from one patient with progressive disease.

Figure 2: T cell reactivity to MCPyV-specific peptides increases after therapy in one patient who had a robust partial response to pembrolizumab. **A)** Cytokine secretion by circulating PBMC to pools of MCPyV-specific peptides was measured from samples obtained immediately pre-treatment and after 12* weeks of pembrolizumab therapy (*for one patient, post-treatment sample was taken 21 weeks post therapy). CD8+ T cells from only two patients secreted IFNg and/or IL-2 in response to these peptide pools; T cells from one patient with a partial response had a significant increase (average of 16 fold) in the frequency of T cells reactive to both pools 1 & 2 after therapy. **B)** The frequency of tetramer+ T cells restricted to an epitope present in Pool 1 increased ~ 7x after therapy. **C)** Reactive T cells were polyfunctional (secreted both IFNg and TNFa), were effector memory T cells (CD45RA-/CCR7-) and expressed PD-1 (56.7% and 76.1% of responding cells were PD-1+). **D**) The patient's confluence of necrotic liver masses decreased in size from 13 cm to 6.4 cm at longest diameter, as visualized by CT scans obtained at baseline and 12 weeks after initiating therapy.

Figure 3: PD-1 expression on circulating CD8+ T cells decreases after therapy and does not correlate with response to pembrolizumab. **A**) We assessed the expression of PD-1 on all circulating CD8 T cells from pre- and post-treatment PBMC (n=11). In 10 of 11 patients, PD-1 positivity on CD8 T cells decreases post-therapy, by a median of 36%. **B**) There was no

association between PD-1 expression on all CD8 T cells pre-treatment and response to pembrolizumab (p= 0.58 by Mann Whitney). **C**) There is a trend toward an increased % of CD8 T cells that have an effector memory phenotype among patients with progressive disease.

Figure 4: Phenotype of MCPyV-specific tetramer+ T cells. **A**) Percentage of MCPyV-specific T cells expressing inhibitory receptors, analyzed directly ex vivo from PBMC two patients with a complete response or disease progression and **B**) density (by MFI) of these receptors. MCPyV-specific T cells from the responding patient have a lower percent positive and lower density of PD-1. **C**) Phenotype of MCPyV-specific T cells compared to CMV-specific T cells over time in a patient with complete response. While expression of the inhibitory receptor 2B4 stays constant on CMV-specific T cells, it decreases on MCPyV-specific T cells.

Figure 5: TCR clonality and T cell fraction within pre-treatment tumors. **A)** TCR clonality is not associated with response to pembrolizumab (p=0.72 by Mann-Whitney test). **B)** TCR Clonality is significantly increased in patients with virus-positive MCCs compared to those with virus-negative MCCs (p=0.0009 by Mann-Whitney test). **C)** The fraction of tumor comprised of T cells (T cell fraction) was not significantly different between patients who did and did not respond to pembrolizumab (p=0.89 by Mann-Whitney test). **D)** There was a trend for increased T cell fraction among virus-positive tumors (p=0.095 by Mann-Whitney test).

Figure 6: Oncoprotein antibody titer was tracked over time in sero-positive individuals with available post-treatment serum samples (n=11). **A)** Of these patients, two had a complete response that was observed on first scan at 12 weeks after initiating pembrolizumab. Among partial responders (n=6), titers also decreased over time in most patients. One patient had an initial partial response but subsequently recurred; this recurrence was preceded by a rise in titer from baseline to week 12 (denoted with *). In another patient with a partial response, a titer

obtained at 3 weeks when a clinical response was noted shows a 2x increase in titer. For patients who did not respond, titer remained constant or increased along with disease burden. **B)** Fold change in oncoprotein antibody titer from baseline to last available sample is depicted for all patients depicted in A; the partial responder who subsequently recurred is denoted with *.

Figure 7: Expression of PD-L1, PD-1 and peritumoral CD8+ cells in pretreatment tumor specimens, as detected by immunohistochemistry, does not correlate with response to pembrolizumab. No significant association between pretreatment expression of PD-L1 on (**A**) tumor-infiltrating immune cells or (**B**) at the tumor perimeter and RECIST response to pembrolizumab was observed (n= 24, p=0.60 or p=1, respectively, by unconditional exact test). Two patients were not included in this analysis: one patient had not yet had response evaluation and one patient's stained tumor specimen was technically inadequate. Expression of PD-L1 on tumor infiltrating immune cells was graded on a 0-3 scale, with 0= absent, 1 = singular infiltrating cells or only very small focus of IC collection at periphery, 2 = focally dense infiltrate along periphery or focal area of diffuse infiltration (IC away from perivascular areas) and 3 = multiple areas of infiltration or diffuse infiltration and/or dense ICs along large stretches of perimeter; samples with a score of 0-1 were binned as PD-L1 low, and samples with a score of 2-3 were binned as PD-L1 high. Perimeter expression of PD-L1 was graded by %; samples with <5% of the perimeter expressing PD-L1 were binned as PD-L1 low. **C**) Tumoral PD-1 expression was graded on a semi-quantitative 0-5 scale; no significant difference was detected between PD-1 expression among patients who did and did not respond to pembrolizumab (p= 0.71 by Mann Whitney test). Two patients were not included in this analysis: one patient had not yet had response evaluation and one patient's stained tumor specimen was technically inadequate. **D)** Peritumoral CD8+ cells (abutting but not infiltrating tumor) were scored on a 0-5 semi-quantitative scale and were not significantly different between patients who did and did not respond to pembrolizumab (p= 0.48 by Mann Whitney test). Three patients were not included in

this analysis: one patient had not yet had response evaluation and two patients' stained tumor specimen were technically inadequate.

Figure 8: There is a trend toward increased expression of PD-L1 and peritumoral CD8+ cells in virus-positive tumors from patients. **A)** Patients with virus-positive tumors were more likely to have high expression of PD-L1 on tumor infiltrating immune cells (p=0.049 by unconditional exact test). This trend was similar but not significant for PD-L1 expression at the tumor perimeter (**B**, p=0.13 by unconditional exact test). **C**) There was no difference between mean PD-1 expression among patients with virus-negative or virus-positive tumors (p=0.86 by Mann Whitney test). **D)** There was trend toward increased score of peritumoral CD8+ T cells among patients with virus-positive tumors (p=0.059 by Mann Whitney test).

Table 1: Tumor MCPyV status, pretreatment MCPyV-specific B and T cell reactivities, and clinical response in 26 patients with MCC receiving pembrolizumab

Patient no.	Tumor viral status *	Antibodies to small T-antigen **	MCPyV tetramer analysis†	Clinical response††
1	+	+	+	CR
2	+	+	+	CR
3	+	+	+	PR
4	+	+	+	PR
5	+	+	+	PD
6	+	+	+	PR
7	+	+	-	PR
8	+	+	-	SD
9	+	+	-	PD
10	+	+	***	PR
11	+	+	***	PR
12	+	+	***	Unconfirmed PR
13	+	+	***	PD
14	+	+	***	PD
15	+	+	***	Not evaluable
16	+	-	***	CR
17	+	-	-	PR
18	-	-	-	CR
19	-	-	-	PR
20	-	-	-	PR
21	-	-	-	PR
22	-	-	-	PD
23	-	-	-	PD
24	-	-	-	PD
25	-	-	***	PD
26	-	-	***	PD

* Patients were considered to have virus-positive MCC if tumors expressed MCPyV Large T-Antigen oncoproteins by IHC (Shuda, Int J Cancer, 2009). Patients with indeterminate tumor IHC results who were positive for serum antibodies or circulating MCPyV-specific T-cells were categorized as having MCPyV-positive tumors (details below).

** Baseline serum samples from all patients were used to measure MCPyV small T-Antigen oncoprotein antibody titers at Laboratory Medicine (University of Washington, Seattle, WA) as described (Paulson KG et al., Cancer Res 2010). Titers above 74 were considered positive (denoted ST+ in table).

†All patients were low-resolution HLA genotyped to determine eligibility for CD8 T cell specific MCPyV peptide-MHC tetramer screening (Bloodworks Northwest, Seattle, WA). Pre- and post-treatment peripheral blood mononuclear cells (PBMCs) collected from patients with HLA-I types that corresponded to available MCPyV-specific tetramers (A*02:01, A*24:02, B07:02, B*35:02, or B37:01; n=17 patients) were stained with appropriate tetramers and analyzed by flow cytometry. Samples with >0.01% of CD8+ T cells co-staining with tetramers were considered positive.

††PBMCs from the first 12 patients with available pretreatment and week 12 post-treatment blood collections were stimulated with pools of MCPyV-specific peptides in a flow cytometry-based intracellular cytokine secretion assay (HIV Vaccine Trials Network, Seattle, WA). PBMCs that secreted interferon-gamma and/or IL-2 robustly (≥0.1% of CD8 T cells after background subtraction) were considered reactive to MCPyV.

*** Nine patients had HLA-I types not amenable to tetramer staining and could thus not be evaluated for the presence of T cells recognizing MCPyV.

Figure 1

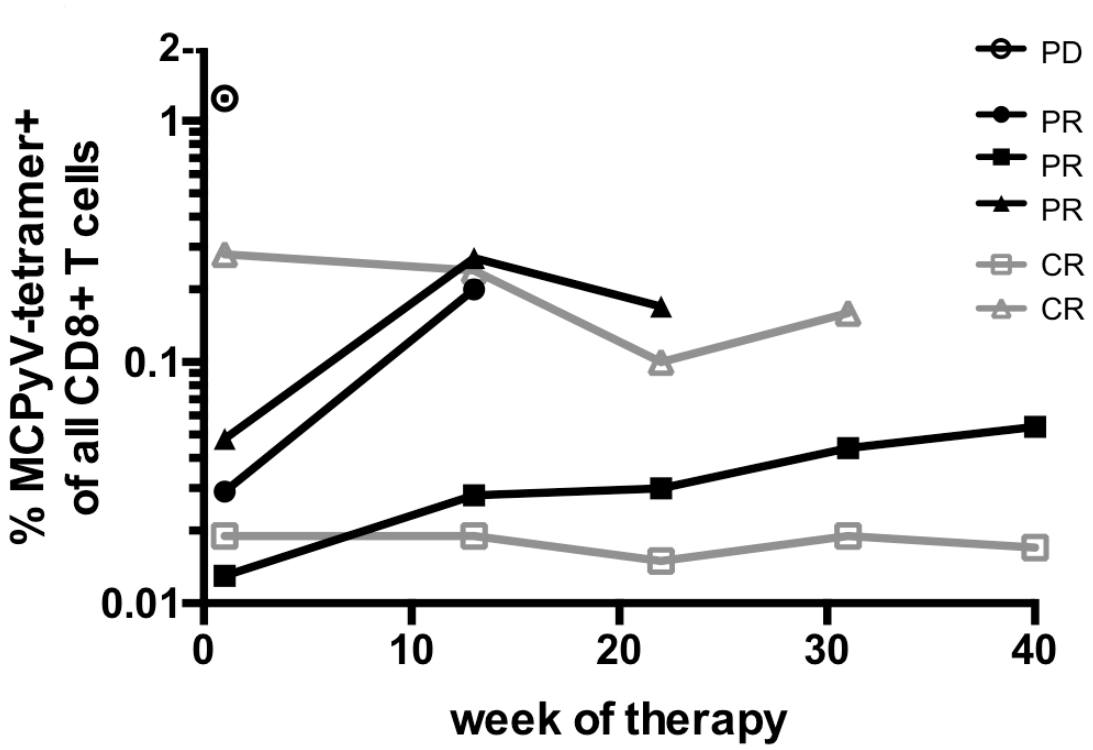
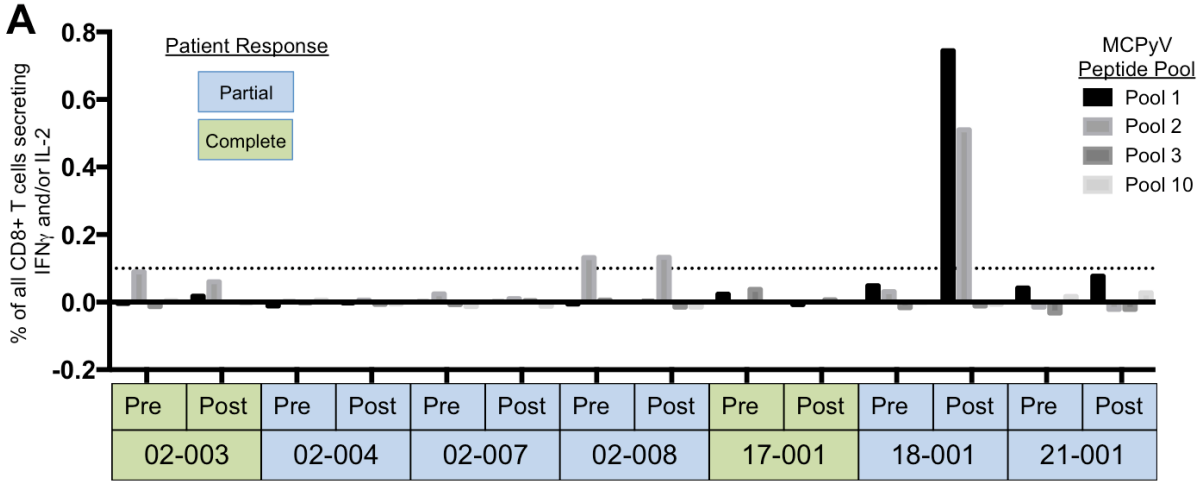
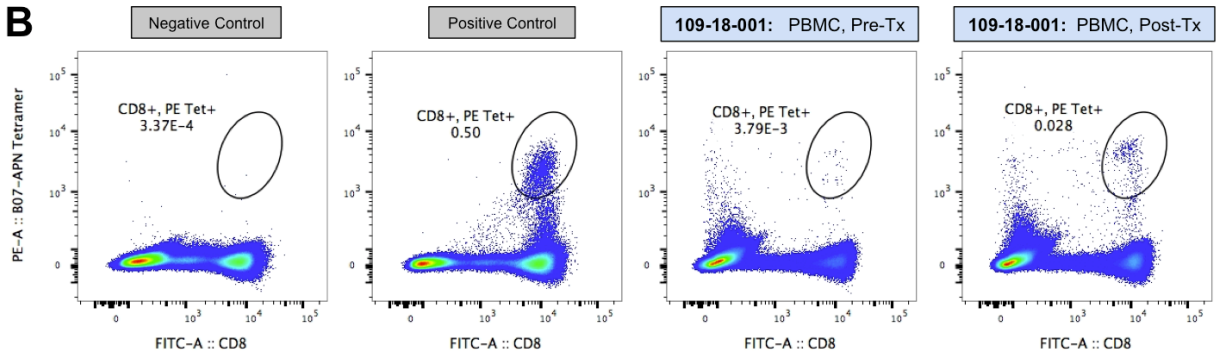
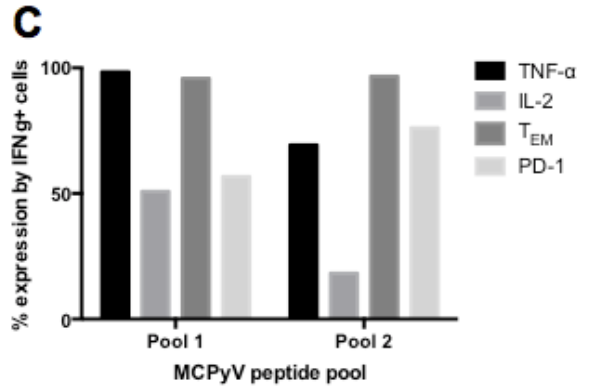

% MCPyV-tetramer+ of all CD8+ T cells
2
1
0.1
0.01
0
10
20
30
40
week of therapy
PD
PR
PR
PR
CR
CR

Figure 2


A
Patient Response
Partial
Complete
MCPyV
Peptide Pool
Pool 1
Pool 2
Pool 3
Pool 10
% of all CD8+ T cells secreting IFNγ and/or IL-2
0.8
0.6
0.4
0.2
0.0
-0.2
Pre
Post
02-003
02-004
02-007
02-008
17-001
18-001
21-001


B
Negative Control
Positive Control
109-18-001: PBMC, Pre-Tx
109-18-001: PBMC, Post-Tx
PE-A :: B07-APN Tetramer
CD8+, PE Tet+
3.37E-4
0.50
3.79E-3
0.028
FITC-A :: CD8


C
% expression by IFNg+ cells
100
50
0
TNF-α
IL-2
TEM
PD-1
Pool 1
Pool 2
MCPyV peptide pool

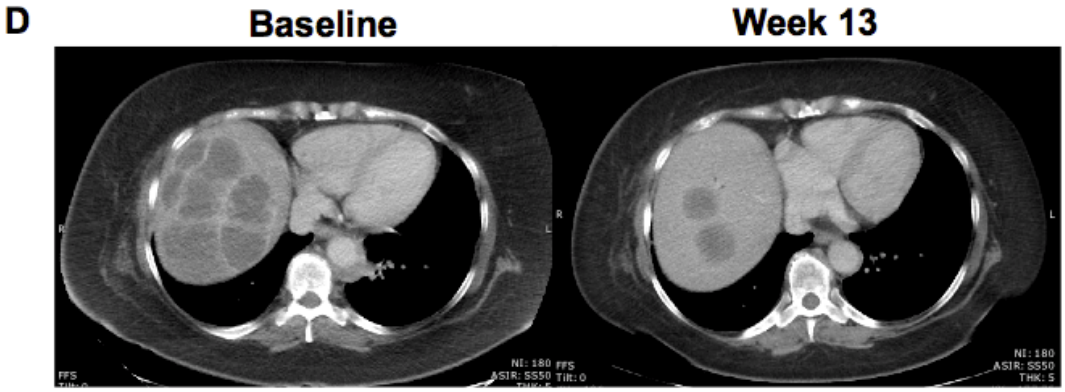

D
Baseline
Week 13

Figure 3

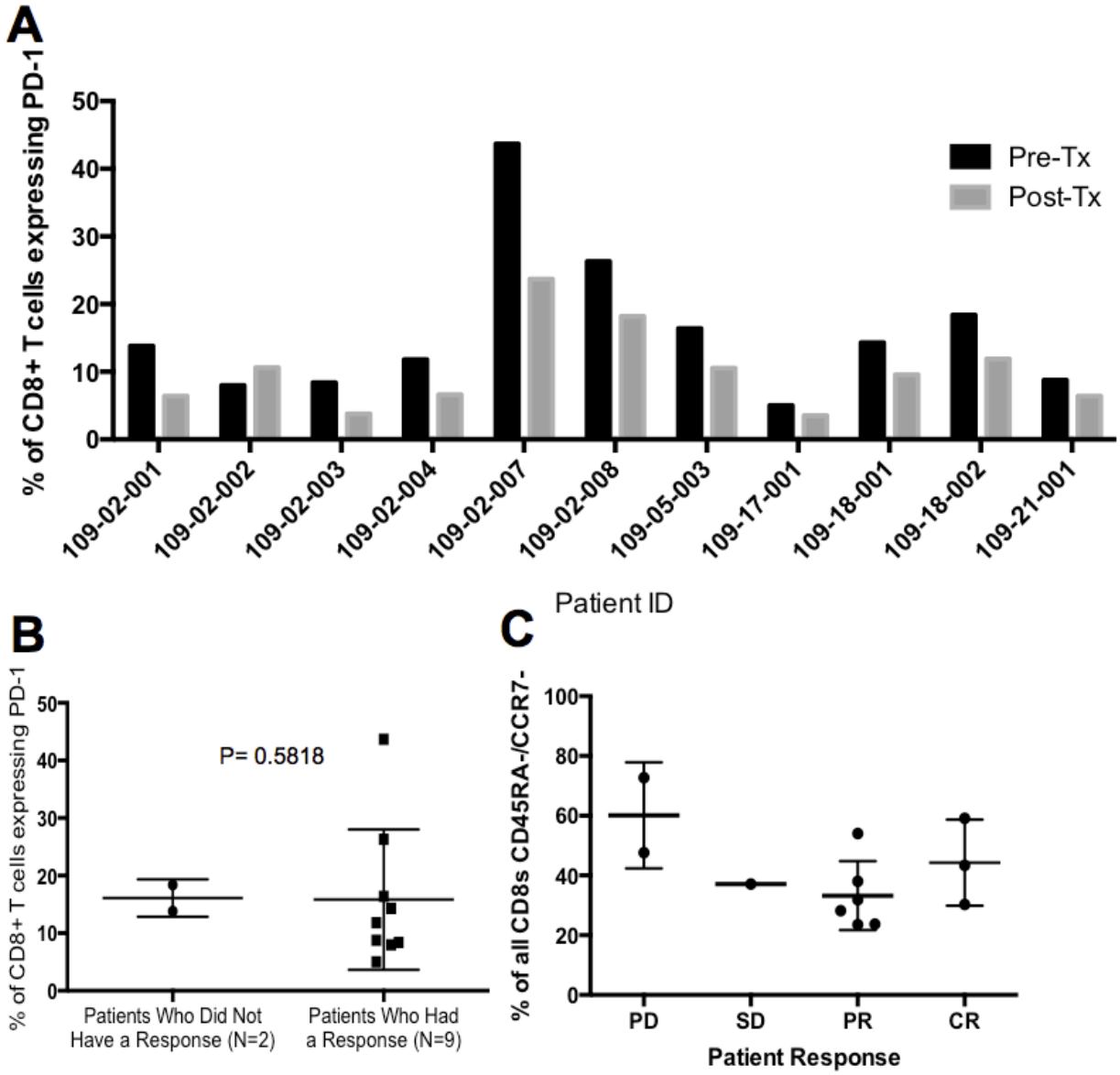

A
% of CD8+ T cells expressing PD-1
50
40
30
20
10
0
Pre-Tx
Post-Tx
109-02-001
109-02-002
109-02-003
109-02-004
109-02-007
109-02-008
109-05-003
109-17-001
109-18-001
109-18-002
109-21-001
Patient ID
B
% of CD8+ T cells expressing PD-1
P= 0.5818
Patients Who Did Not Have a Response (N=2)
Patients Who Had a Response (N=9)
C
% of all CD8s CD45RA-/CCR7-
100
80
60
40
20
0
PD
SD
PR
CR
Patient Response

Figure 4

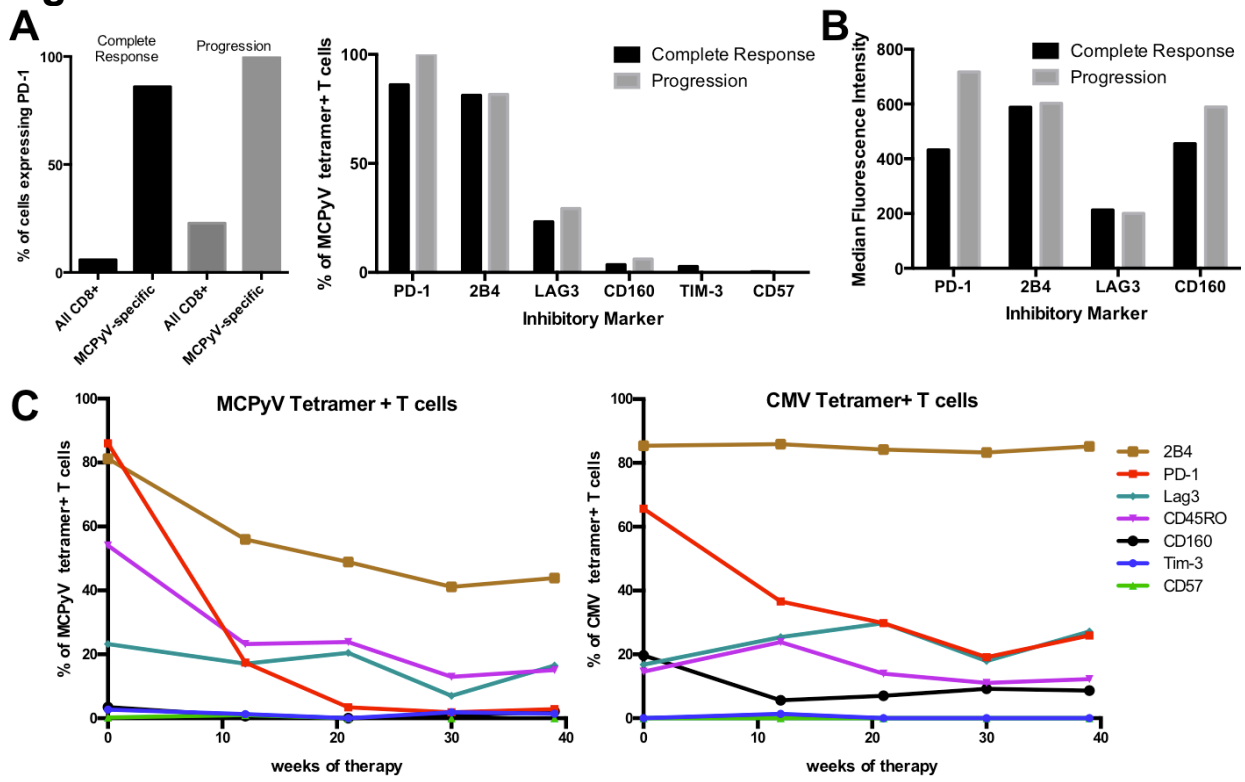

A
Complete Response
Progression
% of cells expressing PD-1
100
50
0
All CD8+
MCPyV-specific
All CD8+
MCPyV-specific
% of MCPyV tetramer+ T cells
100
50
0
Complete Response
Progression
PD-1
2B4
LAG3
CD160
TIM-3
CD57
Inhibitory Marker
B
Median Fluorescence Intensity
800
600
400
200
0
Complete Response
Progression
PD-1
2B4
LAG3
CD160
Inhibitory Marker
C
MCPyV Tetramer + T cells
% of MCPyV tetramer+ T cells
weeks of therapy
CMV Tetramer+ T cells
% of CMV tetramer+ T cells
weeks of therapy
2B4
PD-1
Lag3
CD45RO
CD160
Tim-3
CD57

Figure 5

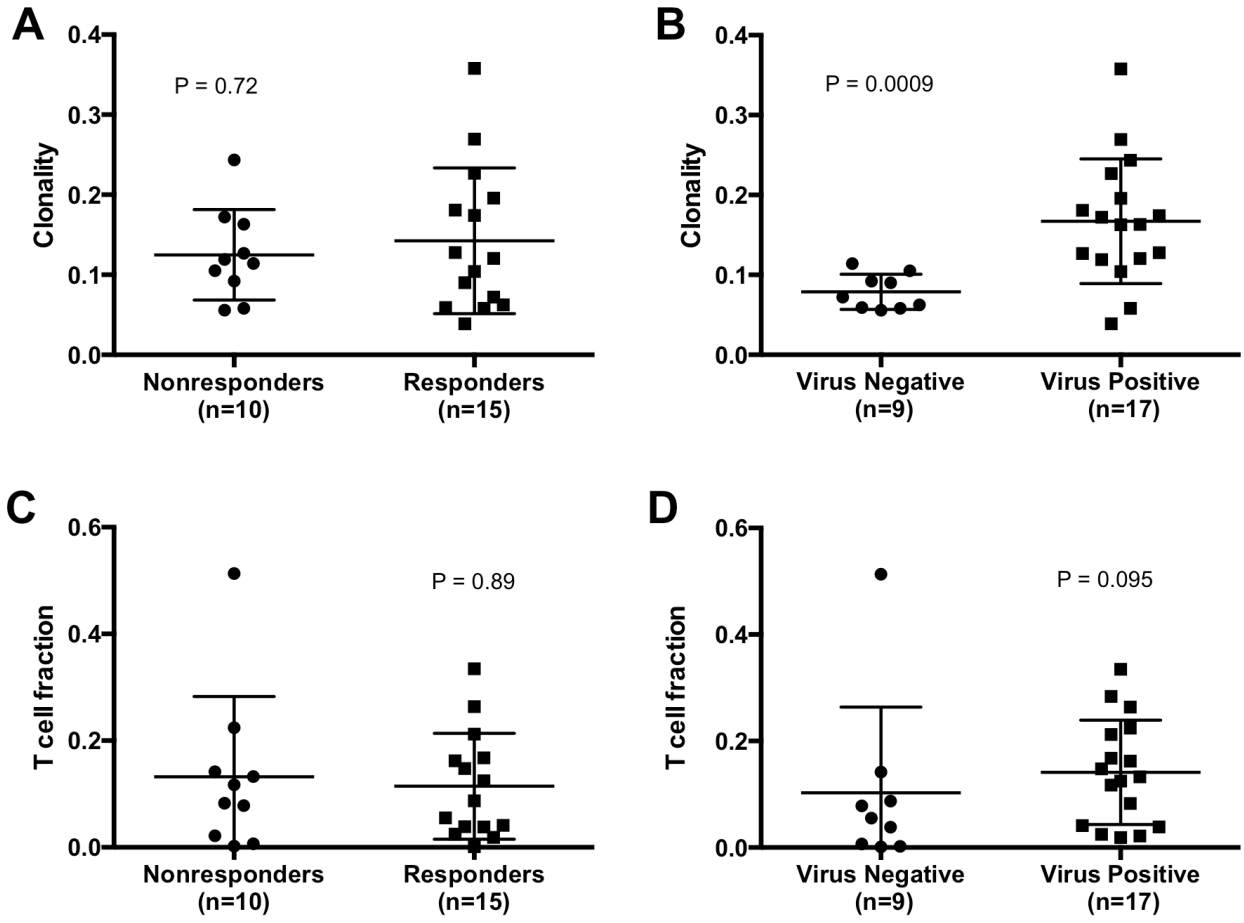

A
Clonality
0.4
0.3
0.2
0.1
0.0
P = 0.72
Nonresponders (n=10)
Responders (n=15)
B
Clonality
P = 0.0009
Virus Negative (n=9)
Virus Positive (n=17)
C
T cell fraction
0.6
0.4
0.2
0.0
P = 0.89
Nonresponders (n=10)
Responders (n=15)
D
T cell fraction
P = 0.095
Virus Negative (n=9)
Virus Positive (n=17)

Figure 6

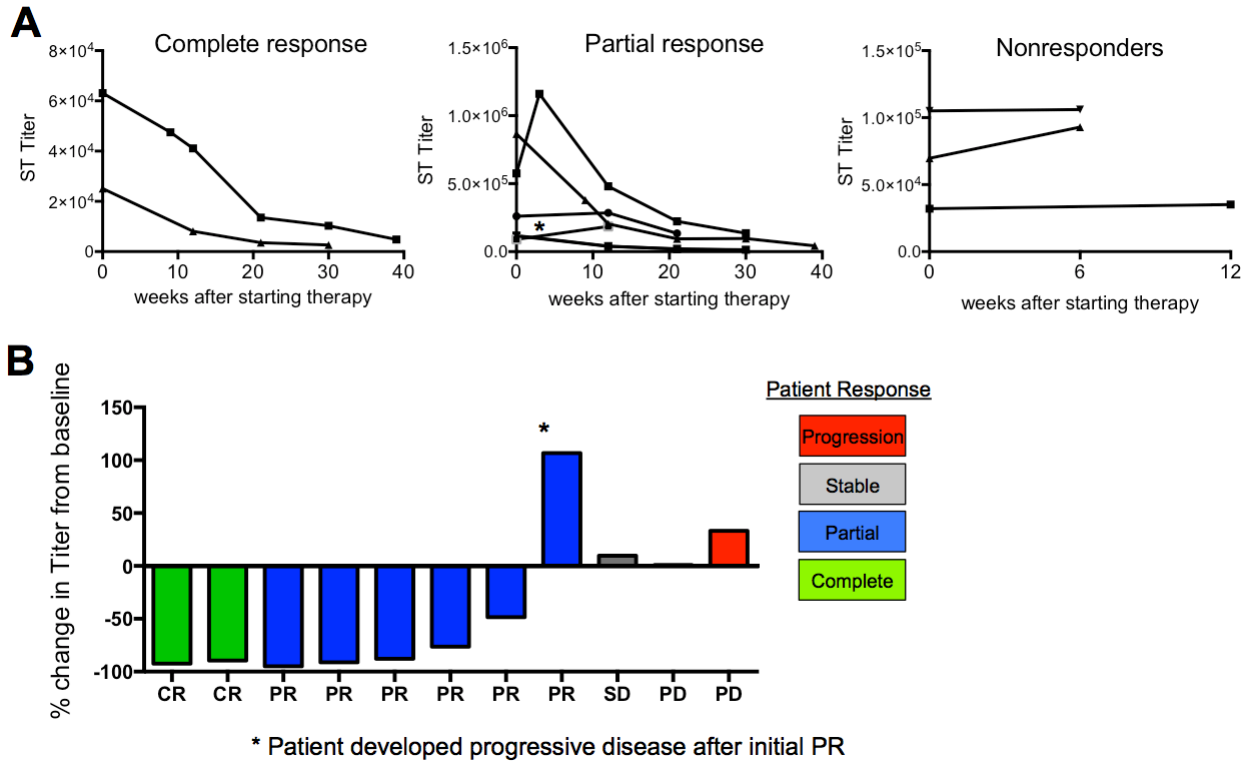

A
Complete response
Partial response
Nonresponders
ST Titer
weeks after starting therapy
B
% change in Titer from baseline
Patient Response
Progression
Stable
Partial
Complete
CR CR PR PR PR PR PR PR SD PD PD
* Patient developed progressive disease after initial PR

Figure 7

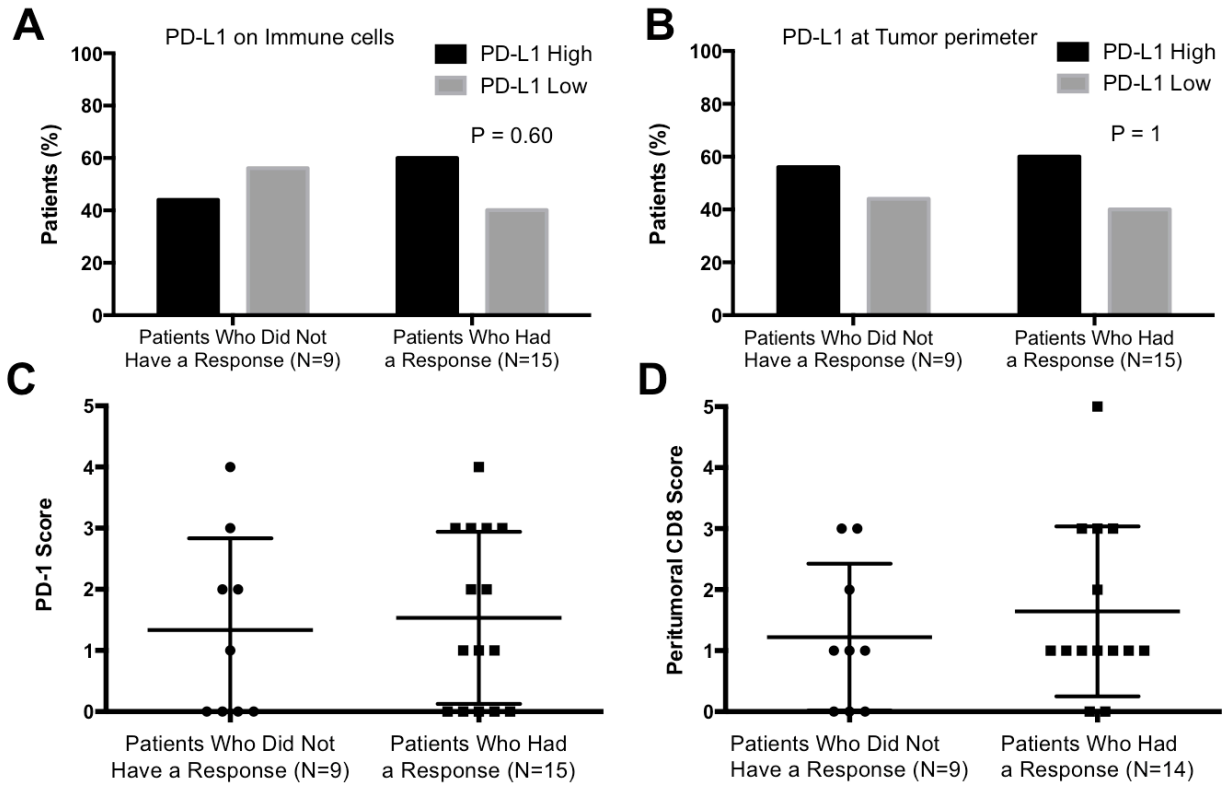
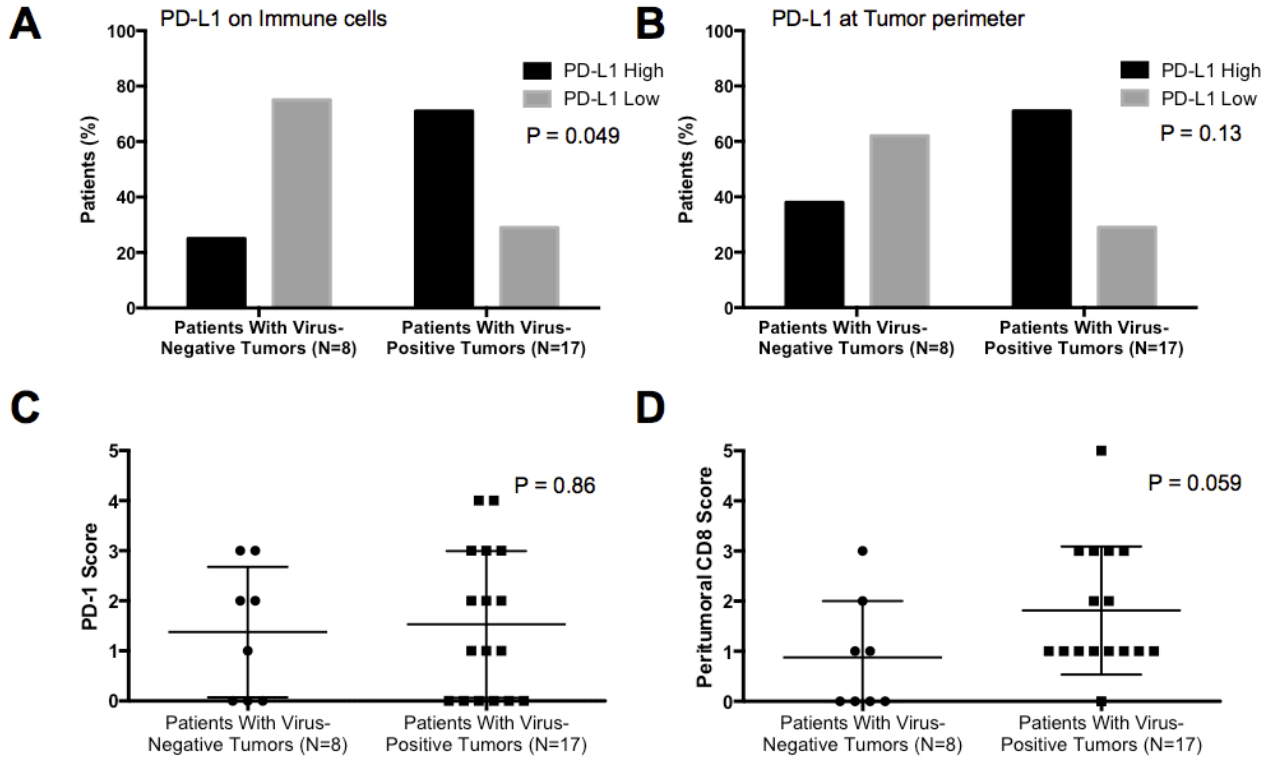

A
PD-L1 on Immune cells
PD-L1 High
PD-L1 Low
P = 0.60
Patients (%)
100
80
60
40
20
0
Patients Who Did Not Have a Response (N=9)
Patients Who Had a Response (N=15)
B
PD-L1 at Tumor perimeter
PD-L1 High
PD-L1 Low
P = 1
Patients (%)
Patients Who Did Not Have a Response (N=9)
Patients Who Had a Response (N=15)
C
PD-1 Score
5
4
3
2
1
0
Patients Who Did Not Have a Response (N=9)
Patients Who Had a Response (N=15)
D
Peritumoral CD8 Score
Patients Who Did Not Have a Response (N=9)
Patients Who Had a Response (N=14)

Figure 8


A
PD-L1 on Immune cells
PD-L1 High
PD-L1 Low
P = 0.049
Patients (%)
Patients With Virus-Negative Tumors (N=8)
Patients With Virus-Positive Tumors (N=17)
B
PD-L1 at Tumor perimeter
PD-L1 High
PD-L1 Low
P = 0.13
Patients (%)
Patients With Virus-Negative Tumors (N=8)
Patients With Virus-Positive Tumors (N=17)
C
P = 0.86
PD-1 Score
Patients With Virus-Negative Tumors (N=8)
Patients With Virus-Positive Tumors (N=17)
D
P = 0.059
Peritumoral CD8 Score
Patients With Virus-Negative Tumors (N=8)
Patients With Virus-Positive Tumors (N=17)

8. Conclusions

Summary of research findings

Since the discovery of the Merkel cell polyomavirus (MCPyV) just 8 years ago[1], a remarkable body of work has been generated to understand the immune response to and immune evasion by this virus-driven cancer. Seminal work by others in the Nghiem lab, including two previous MD/PhD students, elucidated the importance of tumor-infiltrating CD8+ T cells to patient outcomes[23], established many tools to study these MCPyV-specific T cells[24,25], and helped define numerous immune evasion techniques such as downregulation of MHC-I or E-selectin within Merkel cell carcinomas (MCCs)[27,54]. The studies within this thesis have expanded on this previous work to further explore additional mechanisms by which some patients may have more effective immune responses to MCC. In addition, we present early clinical and correlative studies of immune-based therapies for MCC, which are displaying impressive efficacy and are poised to change the way we clinically manage patients with metastatic MCC.

Chapter 1 of this thesis provides a comprehensive summary of the biology of, immune responses to, and immune evasion techniques employed by virus-positive MCC, including the studies mentioned above. As previously mentioned, MCC is an aggressive malignancy without any FDA-approved agents for treatment of metastatic disease. In **Chapter 2**, we review the clinical efficacy of standard-of-care therapies for advanced or recurrent MCC, as well as provide an overview of emerging immune- and mechanism-based therapies. At the time that work was published (February 2013), many of these agents were just beginning to be studied in other cancers, and only a few were being studied in MCC. Excitingly, a tremendous amount of progress has been made in the study of immune-stimulating agents. While many studies are still ongoing and therefore not included in this thesis (such as our clinical trials of adoptive T cell therapy for MCC, electroporation of DNA encoding IL-12, or tumoral injection of a TLR-4 agonist

glucopyranosyl lipid antigen [GLA]), we present the clinical results and correlative studies of therapy with an immune checkpoint inhibitor, PD-1, in **Chapters 5 & 6** of this thesis.

In the first section of original research (**Chapter 3**), we investigate one mechanism of potential heterogeneity of the immune response to MCC by studying the genetic and functional diversity of T cell receptors (TCRs) restricted to one prominent MCPyV epitope. We show that these epitope-specific CD8+ T cells have considerable TCR diversity- indeed, we found almost 400 unique TCRβ clonotypes of MCPyV-specific T cells recognizing this one epitope from just 12 patients, only one clonotype of which was shared in multiple patients. Our hypothesis that infiltration of MCPyV-specific T cells leads to superior tumor control was supported by our findings of increased MCC-specific survival in patients with a greater frequency of intratumoral MCPyV-specific T cells. In addition, we found that more avid MCPyV-specific TCRs are associated with patients who had better control of their MCC. Our findings support further investigation of agents that improve T cell tumor homing and infiltration, as well as use of avid TCRs for transgenic T cell therapy in advanced MCC, discussed in more detail below.

In the next chapter (**Chapter 4**), we performed detailed studies of MCPyV-specific T cells. We found that the frequency of these T cells within PBMC fluctuates with tumor burden. Using multicolor flow cytometry, we phenotyped MCPyV-tetramer+ T cells from blood or tumors of MCC patients. MCPyV-specific T cells more frequently expressed inhibitory markers such as PD-1 and TIM-3, compared to T cells specific for epitopes from other chronic viruses. PD-1 was also expressed at much greater density on MCPyV-specific T cells compare to other virus-specific T cells. We hypothesized that this upregulation of inhibitory receptors resulted in decreased function of MCPyV-specific T cells, but that this phenotype could be reversed. Indeed, we saw that T cell function could be restored by culture *ex vivo,* and that addition of antibodies that blocked either PD-1 or TIM-3 signaling further augmented this response to MCPyV-specific peptides *in vitro*. These studies strongly supported the therapeutic use of antibodies blocking such inhibitory receptor signaling in MCC.

We then transition to the clinical study of emerging therapeutics in MCC. These studies include agents that were proposed as promising in our 2013 review (**Chapter 2**) and have now been evaluated for clinical efficacy. We begin in **Chapter 5** with a retrospective review of single-fraction radiation therapy (SFRT) for metastatic MCC. Therapy with a single, 8 Gray (Gy) dose was hypothesized to have many benefits compared to conventional fractionated radiation (typically a total of 50-60 Gy given in doses of 2 Gy over several weeks). One hypothesis was that radiation stimulates tumor infiltration by immune cells, and that by giving only a single dose of radiation to the tumor, those immune cells would not be harmed by subsequent radiation. We saw an objective response rate of 92% in irradiated lesions, including complete responses in 45% of treated tumors. There was a trend to increased progression among patients with known immunosuppression or prior chemotherapy, which supports our hypothesis that SFRT acts in synergy with the immune system. Importantly, SFRT was well tolerated, offered palliation even of bony metastases, and was convenient for elderly patients.

Excitingly, the field of immune therapy has made significant advances over the last few years, including the FDA-approval of immune checkpoint inhibitors (ICI) such as ipilimumab (anti-CTLA-4) and pembrolizumab (anti-PD-1) for melanoma. These agents act to bind inhibitory receptors expressed on T cells, thus 'releasing the breaks' on the immune system. We hypothesized that therapies that block the PD-1/PD-L1 axis might be particularly effective in MCC, as MCPyV specific T cells express high levels of PD-1 (**Chapter 4**) and the majority of MCCs express its ligand PD-L1 on tumor and immune cells[55,56]. Indeed, as described in **Chapter 6**, we observed an objective response rate of 56% among patients with metastatic MCC treated with the PD-1 inhibitor pembrolizumab as first systemic therapy. Patients with both virus-positive and virus-negative patients responded to pembrolizumab at similar rates. While the trial is still ongoing, responses appear durable with 67% of responding patients remaining progression-free at 6 months- results that already surpass the historical durability of chemotherapy (24% of responding patient progression free at 6 months)[8]. Correlative studies of

pre-treatment tumors showed that there was no association between expression of PD-L1 on tumor cells and response to pembrolizumab. Similarly, there was no significant difference between the degree of intratumoral CD8 infiltration among patients who did and did not respond to pembrolizumab. Further studies to discover a biomarker of response to pembrolizumab, as well as gain insight into the drug's mechanism of action, are ongoing as detailed below.

Future directions of translational research

Expanding efficacy of checkpoint inhibitor therapy

Despite the impressive 56% response rate to PD-1 blockade described in **Chapter 6**, approximately half of MCC patients will not respond to this drug and a subset of responders will be predicted to eventually relapse. To date, no biomarker has been established to predict which MCC patients will or will not respond to PD-1 therapy. Further correlative studies such as those described in **Chapter 7** may contribute to this goal.

In addition, some correlative studies are ongoing or under consideration and not reported in **Chapter 7**. This includes sequencing the repertoire of T cell receptors (TCRs) within pre-treatment tumors of trial patients. In melanoma, response to PD-1 could be predicted by measuring the clonality, or inverse of diversity, of T cells infiltrating each tumor, as determined by measuring the evenness of the TCR infiltrate. Patients who responded to PD-1 had tumors infiltrated by a more oligoclonal population of T cells (increased clonality, indicative of an antigen-specific infiltration)[53]. We are currently analyzing the clonality of all 26 pre-treatment MCC tumors from the PD-1 clinical trial, to determine whether this finding is also observed in MCC. In addition, we are assessing the post-PD-1 T cell infiltrate of tumor biopsies from several responding and non-responding patients via both TCR sequencing and multiplex immunohistochemistry, to measure whether a distinct intratumoral immune response can be correlated with PD-1 responsiveness.

Another candidate correlative study not described in this thesis is to assess gene expression profiles of MCPyV-specific T cells among patients who do and do not respond to therapy, as well as within patients throughout therapeutic course, using methods similar to those described in Han, et al[57].

Lastly, we observed rapid clinical responses to PD-1 after as little as three weeks and one dose of PD-1. However, post-treatment serum and PBMC samples were not obtained until 12 weeks after starting therapy, and therefore we hypothesize that we may have missed the window of maximum B and T cell reactivity in many patients. In the ongoing expansion trial of pembrolizumab in metastatic MCC patients, we will collect and analyze samples at 3 weeks post-therapy. We hypothesize that we may see an increase in oncoprotein titer and/or MCPyV-specific T cells, indicating immune reactivation by pembrolizumab, at this earlier time point.

While we continue to work toward understanding which patients will and will not respond to PD-1 inhibitors, effort is needed to understand which of many other immunotherapeutic agents might best be combined with PD-1, or used for patients who fail PD-1. These immunotherapies include other checkpoint inhibitors that reverse T cell dysfunction such as ipilimumab (anti-CTLA-4), as well as intratumoral therapies such as injection of DNA encoding Interleukin-12 or of glucopyranosyl lipid antigen, a TLR4 agonist. In addition, many other checkpoint inhibitors are being studied in clinical trials. Detailed study of the tumor microenvironment, including quantification of regulatory T cells and myeloid derived suppressor cells (MDSCs), and/or discovery of inhibitory markers upregulated on MCPyV-specific T cells, may provide critical insight into which agents should be added in combination with PD-1 therapy in certain patients. Importantly, study of blood and tumor samples from patients responding to combination therapies may elucidate novel mechanisms of synergy between agents, and may identify treatment strategies that could also work well for additional cancer types.

An example of successful combination therapy is illustrated by a case report on one patient with metastatic MCC who received pembrolizumab in combination two infusions of MCPyV-

specific T cells as part of our adoptive T cell clinical trial. Despite therapy, this patient developed progressive disease. After ipilimumab was added to his therapeutic regimen (~5 months after initiating PD-1 + T cell therapy), he experienced a robust partial response (86% reduction in tumor volume) that is ongoing 10 months after first dose of ipilimumab. We are currently studying the phenotype (inhibitory receptor expression and effector function) and TCR repertoire of his circulating and tumor-infiltrating MCPyV-specific T cells throughout his therapeutic course to try to understand why ipilimumab, but not pembrolizumab, was more effective at stimulating this patient's immune system.

Adoptive T cell transfer and development of transgenic TCRs

One additional therapy that holds promise for MCC and could be used for patients who either fail ICI or are ineligible for ICI due to pre-existing immunosuppression is the generation of transgenic therapeutic T cells. Though not discussed in detail in this thesis, we are currently executing a Phase I/II Clinical Trial to treat MCC patients with autologous T cell therapy (NCI R0ICA176841, P. Nghiem, PI). Among the initial four patients treated with MCPyV-specific T cells alone, limited responses were seen (one patient had a complete response but subsequently recurred, two patients had partial responses, one patient had progressive disease). Addition of an ICI, anti-PD-L1, to the trial has increased efficacy and elicited complete responses in the two patients who have received this combination therapy. However, as evidenced by the above patient vignette, some patients will still not respond to PD-1/PD-L1 axis blockade + adoptive T cell therapy.

A strategy that may increase the efficacy of T cell therapy would be to create T cells transgenic for an avid MCPyV-specific TCR. A great body of literature has suggested that the avidity of the TCR is linked to efficacy of the T cell effector function *in vivo*[40-43]. Our preliminary study of the avidity of endogenous MCPyV-specific TCRs also suggests that patients with more avid TCRs may have better outcomes (**Chapter 3**). In addition, we have found that of all

HLA*A02+ patients in our cohort, a minority (~14%) have detectable HLA*A02-restricted MCPyV-specific tetramer + T cells in their PBMC. Based on these findings, we have proposed to generate transgenic MCPyV-specific T cells with an avid HLA*A02-restricted TCR (tTCRs) isolated from a patient with an impressive immune response to MCPyV.

There are numerous potential benefits to tTCRs beyond conventional adoptive T cells. These benefits include the ability to treat people who lack endogenous T cells, such as the ~86% of HLA*A02+ patients who lack detectable MCPyV-specific T cells. In addition, even for patients who have an existing MCPyV-restricted T cell population, these tTCR T cells may be more avid than their existing effector T cells. Lastly, in generating these tTCR T cells we can select a less differentiated population of T cells to reprogram (ie, naïve or T_{CM} cells) that would likely be less 'exhausted' than the endogenous MCPyV-specific T cells and persist longer after infusion. We are well on our way to providing proof-of-concept for this strategy, after successfully identifying multiple avid TCRs (**Chapter 3**). These TCRs will be tested *in vitro* as well as in pre-clinical mouse models as we develop their use for patients.

Enlisting 'help' from CD4+ T cells

Another promising strategy to increase the efficacy of adoptive CD8+ T cell therapy would be to increase the support provided to CD8+ T cells by CD4+ T cells. There is considerable evidence that CD4+ T cells, specifically Th1/Th17-skewed CD4+ T cells, support the survival, cytotoxicity, proliferation, and tumor infiltration of CD8+ T cells in other tumor models[58-60] and that adoptive T cell therapy with both CD4+ and CD8+ T cells can mediate superior outcomes to CD8+ T cells alone[61]. MCPyV-specific CD4+ T cells recognizing at least three distinct epitopes can be identified and tracked in patients with either of two HLA-II types present in ~20% of the population[24](Natalie Vandeven, unpublished observations). Therapeutic MCPyV-specific CD4+ T cells recognizing these epitopes could be generated and infused along with CD8+ T cells in patients with appropriate HLA-I and HLA-II types. Alternatively, an avid

HLA-I restricted TCR that is 'CD8-indepentent' (ie, does not require stabilization by CD8 for binding to pMHC) could be transduced into both CD8+ and CD4+ T cells from a patient, allowing patients with any HLA-II type to be treated with both types of T cells. Many of such CD8-independent TCRs were discovered in **Chapter 3**, highlighting the feasibility of this approach.

Identification of Neoantigen-specific T cells

While we have focused predominantly on virus-positive MCC due to the ease of studying T cells specific for MCPyV, excitingly, we are just beginning to elucidate the biology behind the ~20% of MCCs that are not caused by MCPyV. Historically, patients with virus-negative MCC have had a poorer prognosis compared to virus-positive MCCs[62,63]. Whole exome sequencing of MCCs has revealed that on average, these virus-negative MCCs have ~100x greater load of somatic single nucleotide variants (SSNVs) than virus-positive MCCs[64]. In fact, virus-negative MCCs have, on average, more mutations than any other cancer studied including other UV mutation-induced cancers such as melanoma. Often, these SSNVs encode neoantigens that can be recognized by the immune system as foreign be targeted by cytotoxic T cells, as has been observed for other cancers[65,66]. Excitingly, recent publications have highlighted cases of impressive clinical responses of patients with melanoma and NSCLC to immune stimulation with checkpoint inhibitor therapy, while demonstrating that neoantigen-specific T cells had expanded and likely contributed to this response[66-68].

These findings mirror our preliminary results in MCC. As described in **Chapter 6,** many virus negative patients had impressive responses to therapy with the checkpoint inhibitor anti-PD-1. In addition, though not discussed in this thesis, the agent PD-L1, which acts similarly to block the PD-1/PD-L1 axis and restore T cell function, was also studied formally in a clinical trial of advanced MCC patients. One virus-negative patient who had progressed while receiving chemotherapy (cisplatin and etoposide), experienced a remarkable response to the PD-L1 drug

after just four weeks (decrease in liver metastasis by 50%) and has remained in response 17 months after enrollment. This patient's whole exome sequencing revealed thousands of tumor-specific SSNVs. Mutated peptides that were thought to be immunogenic (predicted to bind well to her HLA-I type) were tested against the patient's PBMC. Four neoantigen peptides were identified that elicited a specific response from PBMC; responses were not detected against the WT peptide analogs (Candice Church, unpublished observations). Subsequent work determined that these neoantigens were recognized by CD4+ T cells. Ongoing studies aim to create HLA-II tetramers to allow phenotyping and tracking of these T cells over time in this patient. We hope to replicate these studies with PBMC and TIL from other virus-negative patients who are or are not responding to immunotherapy to further understand the T cell correlates of protection. Ultimately, in a true illustration of personalized medicine, tumor neoantigen-specific peptide vaccines could be created to help augment the immune response in these patients.

In conclusion, we have presented detailed studies of the CD8+ T cell response to MCC, a rare but aggressive skin cancer. Both virus-positive and virus-negative MCC can be immunogenic and provide opportunities for study of MCC-specific T cells; in addition, we now understand that both of these MCC subtypes may respond well to immune-based therapies. We have shown promising results that immunotherapies such as anti-PD-1 can provide efficacy for a large fraction of patients, and we anticipate that these results will be followed by FDA-approval of this agent for advanced MCC in the near future. For the first time, options beyond chemotherapy exist for patients with metastatic disease. Ongoing work, stemming from the molecular studies detailed in this thesis, will hopefully provide direction on how to further increase the efficacy of existing therapies.

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